JAN 22 1917
UNIV. OF MICH. LIBRARY

VOLUME II NUMBER 1

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

JANUARY, 1917

EDITOR-IN-CHIEF
C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR

PUBLISHED BI-MONTHLY
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

THE CAMBRIDGE UNIVERSITY PRESS
FETTER LANE, LONDON: E. C.

Entered as second-class matter April 17, 1916, at the Post Office at Baltimore, Maryland, under the Act of March 3, 1879

BACTERIOLOGICAL PEPTON

Fairchild Bros. & Foster

OFFER to the bacteriologist a Pepton which is perfectly serviceable for the formulas and in all the technic of the bacteriological and antitoxin laboratory. It is employed in the usual proportions and for whatever purposes Pepton of this most desirable quality is required.

It may be mentioned that this product is offered at a price which is intrinsically low, in view of the completely serviceable quality of this Pepton for all bacteriological purposes.

PEPTON, FAIRCHILD

PEPTON, Fairchild, is put up in 30 gram vials; in bottles—quarter, half and one pound. May be obtained from dealers in bacteriological supplies.

We shall be pleased to send for trial, a sample sufficient for 1000 cc. of media.

Fairchild Brothers and Foster

New York

CONTENTS

NUMBER 1, JANUARY, 1917

The Colorimetric Determination of Hydrogen Ion Concentration and its Applications in Bacteriology. I. William Mansfield Clark and Herbert A. Lubs 1
Soil Flora Studies. I. H. J. Conn 35
The Influence of Milk and Carbohydrate Feeding on the Character of the Intestinal Flora. IV. Diet versus Bacterial Implantation. Thomas G. Hull and Leo. F. Rettger 47
Coccidia in Subepithelial Infections of the Intestines of Birds. Philip B. Hadley 73
A Bacterial Disease of the Wragg Cherry. Preliminary Note. Walter G. Sackett 79

NUMBER 2, MARCH, 1917

A Study of the Diphtheroid Group of Organisms with Special Reference to their Relation to the Streptococci. I. Characteristics of a Peculiar Pleomorphic Diphtheroid. Ralph R. Mellon 81
The Colorimetric Determination of Hydrogen Ion Concentration and its Applications in Bacteriology. II. William Mansfield Clark and Herbert A. Lubs 109
Soil Flora Studies. II. H. Joel Conn 137
Studies in the Nomenclature and Classification of the Bacteria. II. The Primary Subdivisions of the Schizomycetes. R. E. Buchanan 155
The Evolution and Relationship of the Great Groups of Bacteria. I. J. Kligler 165
A Study of Five Members (or So-called Species) of the Septicemia Hemorrhagica (Pasteurella) Group of Organisms with Special Reference to their Action on the Various Carbohydrates. A. M. Besemer 177
The Large Numbers of *Bact. abortus var. lipolyticus* which may be Found in Milk. Alice C. Evans 185
Creatinine Production by *B. coli* and *B. typhi*. H. J. Sears 187

NUMBER 3, MAY, 1917

The Colorimetric Determination of Hydrogen Ion Concentration and its Applications in Bacteriology. III. William Mansfield Clark and Herbert A. Lubs 191
The Use of a Three Per Cent Lactose Litmus Agar Plate for the Demonstration of *B. coli* in water examination. E. M. Meyer 237
A Simple Method for Staining the Capsules of Bacteria. F. M. Huntoon 241
A Curious Accident Due to *B. pneumoniae*. J. P. Simonds 245

326546

The Fallacy of Refined Readings of Gas Percentages in the Fermentation of Lactose Peptone Bile and Lactose Broth. William W. Browne and Benjamin Ackman 249
Note on the Reaction of the Paratyphoids, Alpha and Beta, in Litmus Milk. Philip B. Hadley 263
A Study of the Diphtheroid Group of Organisms with Special Reference to its Relation to the Streptococci. II. Classification of the Diphtheroid Group. Ralph R. Mellon 269
The Fuchsin-Aldehyde Reaction on Endo Medium. George G. DeBord 309

NUMBER 4, JULY, 1917

The Importance of Uniform Culture Media in the Bacteriological Examination of Disinfectants. J. H. Wright 315
Studies on the Nomenclature and Classification of the Bacteria. III. The Families of the Eubacteriales. R. E. Buchanan 347
The Effect of Hydrogen Ion Concentration on the Production of Precipitates in a Solution of Peptone and its Relation to the Nutritive Value of Media. I. J. Kligler 351
Observations on the Types of Organism Isolated from Water after Treatment with Calcium Hypochlorite. M. A. Smeeton 355
The Morphology of a Strain of *B. diphtheriae*. P. G. Heineman 361
A Note on the Preparation of Hyphomycetes for Microscopical Examination. Mark F. Boyd 365
Blastocystis hominis: Its Characteristics and its Prevalance in Intestinal Content and Feces in South Carolina. Kenneth M. Lynch 369
Characteristics of Coli-like Microorganisms from the Soil. B. R. Johnson and Max Levine 379
The Effect of Sterilization upon Sugars in Culture Media. Courtland S. Mudge 403
The Presence of the *B. coli* and *B. Welchii* Groups in the Intestinal Tract of Fish (*Stenomus chrysops*). William W. Browne 417
Nitrogen Assimilating Organisms in Manure. H. L. Fulmer and E. B. Fred 423
New Differential Plating Methods for *B. bifidus* (Tissier) and *B. acidophilus* (Moro). John C. Torrey 435
Trichomonasis of Chicks: A New and Highly Fatal Disease. John Weinzirl 441
A Study of the Diphtheroid Group of Organisms with Special Reference to its Relation to the Streptococci. III. Immunological Relations. Ralph R. Mellon 447

NUMBER 5, SEPTEMBER, 1917

Frontispiece—Professor H. W. Conn.
Professor H. W. Conn. W. M. Esten 501
The Families and Genera of the Bacteria. Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types. C.-E. A. Winslow, Chairman, Jean Broadhurst, R. E. Buchanan, Charles Krumwiede, Jr., L. A. Rogers, and George H. Smith 505

Counting the Living Bacteria in Milk—A Practical Test. W. D. Frost 567
Studies on the Bacterial Metabolism of Sulfur. I. Formation of Hydrogen Sulfide from Certain Sulfur Compounds Under Aerobic Conditions. Fred W. Tanner 585

NUMBER 6, NOVEMBER, 1917

Early Instructors in Bacteriology in the United States. David H. Bergey 595
Studies on the Nomenclature and Classification of the Bacteria. IV. Subgroups and Genera of the Coccaceae. R. E. Buchanan 603
Bacterium Pyogenes and its Relation to Suppurative Lesions in Animals. Archibald R. Ward 619
Influence of Hydrogen Ion Concentration of Medium on the Reproduction of Alfalfa Bacteria. E. B. Fred and N. E. Loomis 629
The Colon-aerogenes Group from Silage. O. W. Hunter 635
Index 641

THE COLORIMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION AND ITS APPLICATIONS IN BACTERIOLOGY[1]

PART I

WILLIAM MANSFIELD CLARK AND HERBERT A. LUBS

From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C.

Received for publication July 27, 1916

SECTION I. INTRODUCTION

In the classic work of Pasteur are to be found many important suggestions as to the manner in which microbial life responds to the acidity of culture media. As an instance we may recall the opening chapter of Studies on Fermentation, in which Pasteur (1879) notes that the relatively high acidity of must favors a natural alcoholic fermentation in wine, while the low acidity of wort makes the brewing of beer more difficult. Likewise, according to Pasteur, it is the difference in the acidity of wort and must which determines in large measure their relative susceptibility to "disease." Today in the manufacture of beer, certain of the processes are being controlled by following the hydrogen ion concentration; and in judging the quality of wines the determination of their hydrogen ion concentrations is considered an important test.

In his criticism of Duval's theory that yeasts may metamorphose to "lactic ferments," Pasteur (1879), with characteristic glee, pointed out that in Duval's experiment the medium had a reaction favorable to the "lactic ferments" and unfavorable to the yeasts. Consequently, had the inoculation been mixed, the "lactic ferments" would have outgrown the yeasts. Although the direct bearing of this critique is now only historical, the

[1] Published by permission of the Secretary of Agriculture.

remark itself has lost none of its significance. Dalyell (1914) says,

> A constant difficulty in bacteriological examination of feces is the selective action of artificial media which allows certain organisms to outgrow others. For example, if a portion of fecal material be inoculated (a) into ordinary nutrient bouillon of alkaline reaction, (b) into such bouillon containing a large amount of bile, and (c) into nutrient bouillon acidified with 1 per cent of acetic acid, and if the sediments of the three tubes be examined, each will show a different microscopic picture, and none will conform to that of a smear made directly from feces.

Even the influence of acidity upon the effective temperature of the process we have come to call pasteurization did not escape the notice of Pasteur (1879). In a brief paper presented at the Philadelphia meeting of the Society of American Bacteriologists in 1914, one of us (Clark 1915 a) remarked that in cellular destruction temperature is to be considered as an accelerating *condition*, and showed that, among the active *agents* concerned, the concentration of the hydrogen ions in a medium may be of great significance. In a broad sense the term "thermal death point," as Pasteur showed, has no meaning when considered apart from the agents or the processes involved in cellular destruction at high temperatures, although it may have a definite practical significance in the study of such problems as the pasteurization of a relatively constant medium such as milk.

Since Pasteur's time, the influence of acidity and alkalinity upon the activity of microörganisms has been studied in many details and from many points of view. An adequate review of the literature bearing upon this subject is a task for which we must admit our incapacity, for so many of the significant observations which we have noted were gleaned from papers on other themes that we feel sure we have overlooked much truly important work. To deal only with those investigations which have been undertaken with the more modern physical chemist's point of view would be to a certain extent unjust, for many of these are of less real value than the observations made from other view points. A judicious review would also make exorbitant

demands upon space, since many aspects of the subject are worthy of sympathetic treatment. For instance, in considering the introduction of the titrimetric method of adjusting the "reaction" of culture media, it should not only be pointed out that the procedure was applied to a problem where it found no fundamental theoretical justification; it should also be recalled that this method did make possible a certain degree of uniformity in the adjustment of important and well studied media, and that the mere introduction of a scale, however inadequate its unit, was an advance.

To realize the scope and diversity of but one class of problems in which the acidity of culture media is involved, we need only recall those numerous cases in which the acid produced by one organism, has, or is alleged to have, an inhibitory effect upon other organisms. The use of vinegar as a food preservative is essentially a case in point. Vinegar is protected from the successful invasion of the hosts of the microscopic world by its high acidity, and there is no reason to doubt that less acid liquids, though fortified less strongly, resist invasion in precisely the same way. Where indeed shall we place the line of demarcation? Fruit juices, because of their acidity, are perhaps as effectively immune to successful attack by *certain* organisms as the strongest vinegar; and the acidity of cheese probably protects it against those kinds of bacterial action that would make it unfit for human food. The suppression of putrefactive, pathogenic and coliform organisms (in laboratory cultures) by bacteria of the acidophylic bulgaricus type has been widely discussed—very largely because it forms a basic argument for the Metchnikoff treatment of certain intestinal conditions, but also because it is intimately associated with the manufacture of cheese, rennet, silage and several acid fermented foods in which organisms of the bulgaricus type are dominant.

We need not discuss the conflicting conclusions which have been reached in the study of these and similar problems, but we may note that almost without exception the study of the influence of acidity upon microbial activity has been approached with no other method than the determination of "titratable

acidity." The fundamental objections to this method when applied to physiological problems have been pointed out by Henderson (1909), Sörensen (1909 a and b), Michaelis (1914 b) and others, and with special reference to bacteriological culture media by Clark (1915 e).

It may be reiterated here that the titrimetric method, designed originally for the quantitative estimation of strong acids and bases, can not be applied to complex mixtures of very weak acidic and basic groups such as are found in the constituents of most culture media. In so far as the method is used to determine the "free acid" or to adjust to a certain degree of "free acid" it is an absolute failure when applied to culture media.

There is however, an even more fundamental reason why the titrimetric method is inappropriate. Two media adjusted to the same "degree of titratable acidity" may have widely divergent hydrogen ion concentrations as shown by Clark (1915 e). Now, if it is granted that the bacterial enzymes are comparable with other better known enzymes, and, if the validity of such classical work as that of Sörensen (1909) is admitted, then it must be supposed that the hydrogen ion concentration of a medium rather than the titratable acidity influences the enzymatic activity of bacteria and their relatives. If, furthermore, it is granted that the protoplasmic structure of the bacterial cell, or of its membrane, responds to any extent like protein solutions, then there is reason to suppose that the stability of this structure is to some degree dependent upon the hydrogen ion concentration about it. If the membrane is to any degree a mosaic structure with interfaces as sensitive to "reaction" as are those of certain emulsions, then the hydrogen ion concentration of the media must be taken into consideration when dealing with the penetration of food or poison.

Such considerations furnish a more fundamental point of view from which to regard in a new light many of the problems of bacteriology. Using such a view as a working hypothesis, it might safely be postulated that there should be hydrogen ion concentrations which limit the activity of one or another organism. A few of the earlier papers touched on this side of the

subject. Lazarus (1908), for instance, adjusted her media with the aid of various indicators and determined roughly the hydrogen ion concentrations which limited bacterial growth.

In 1912 Michaelis and Marcora, with more accurate methods and a clearer conception of the problem, demonstrated that *B. coli* is limited in its acid fermentation of sugars upon reaching a rather definite hydrogen ion concentration.

A more extensive research by one of us (Clark 1915 c) confirmed the conclusion of Michaelis and Marcora in its essential point. It was mentioned at the time that a similar phenomenon had been observed with a few cultures of streptococci. This has since been abundantly confirmed by Ayers (1916) in his study of some 200 cultures. These results with bacteria of the colon and streptococcus groups together with some preliminary work with other groups of organisms have shown that by determining the final hydrogen ion concentrations of sugar-fermenting cultures, instead of titrating such cultures, values are obtained which are far more constant, and more reproducible in different media, of great diagnostic worth, and of auspicious significance.

A further instance of the use of hydrogen ion determinations is shown in the development of the method of Clark and Lubs (1915) for the differentiation of the two main groups of the colon-aerogenes family.

Before this work on the limiting hydrogen ion concentrations in acid fermentations, Michaelis (1911) had shown that certain organisms, notably *B. typhi*, respond in much the same manner as do proteins and agglutinate within certain zones of hydrogen ion concentration with characteristic optima.

The influence of hydrogen ion concentration upon the activity of yeasts has received considerable attention of late, especially in connection with the manufacture of beer (Emslander, 1914; Lüers, 1914; Leberle and Lüers, 1914; Lüers and Adler, 1915; Hermann; R. Wahl, 1915), wine (Paul, 1914, 1915) and bread (A. Wahl, 1915; cf. Jessen Hansen, 1911). Its influence in yeast culture has been recently reviewed by Hägglund (1915 a and b).

The influence of hydrogen ion concentration on molds has received less attention, if we except some excellent studies of

the mold enzymes. Waterman's (1915) estimate that the critical limit for *Penicilium glaucum* is about 1×10^{-6} N. H and for *Aspergillus niger* about 4.5×10^{-3} N. H, we can not understand. It may be that he has confused Friedenthal's (1910) gram per cubic centimeter basis of hydrogen ion concentration with the generally accepted gram per liter standard. Dr. Currie of this laboratory has cultivated for us an organism which Dr. Thom has classified as an *Aspergillus niger*. When grown on a medium consisting of 1 gram KH_2PO_4, 3 grams $NaNO_3$, 0.5 gram $MgSO_4$ $7H_2O$, 100 grams sucrose, in 1000 cc. water, the medium taken from below the mycelium on the seventh day of growth had a hydrogen ion concentration of 2×10^{-2} N.

Whether different hydrogen ion concentrations have any effect upon the metabolism of molds will constitute an important part of the investigations on soft cheeses which are being carried on in this laboratory.

So numerous have become the researches demonstrating the importance of knowing or controlling the hydrogen ion concentration of biochemically active solutions that the subject is almost too large for review. The main outlines and a review of the more important literature up to 1914 will be found in the invaluable monograph by Michaelis (1914 b) or in the earlier classic papers of Sörensen (1909, 1912). We may, however, take this occasion to mention a few topics which are of special interest to the bacteriologist. Among the studies of the influence of hydrogen ion concentration upon enzyme action researches on the enzymes of yeasts and molds have occupied a prominent place. The bacterial enzymes have received less attention, although Meyer in 1911 determined the influence of hydrogen ion concentrations upon the proteases of *B. prodigiosus* and *B. pyocyaneus*. Itano (1916), in his recent paper on the relation of hydrogen ion concentration of media to the proteoclastic activity of *B. subtilis*, *Strept. erysipelatos* and *Strept. lacticus*, has given some further information; but his references indicate that others have not given this subject the attention it deserves.

Among the enzyme studies of particular interest to bacteriologists is that of Palitzsch and Walbum (1912) on tryptic gelatin liquefaction.

Of almost equal importance in special fields of bacteriological research have been the numerous studies of the influence exercised by the hydrogen ions upon the physical condition of colloids and more particularly upon protein solutions. Correlated with these investigations are the studies on acid agglutination of bacteria (Michaelis 1911, Beniash 1911, and others) and their constituents (Arkwright 1914).

In the work of Jordan (1903), of Atkin (1911, 1914) and of Walbum (1915) upon various phases of haemolysis, and of Hamburger and Hekma (1908) and of Koltzoff (1914) upon phagocytosis, in the voluminous literature upon absorption phenomena, and cell permeability, and in the researches of Hildebrand (1913) and others upon inorganic solutions the influence of the activity of hydrogen ions has been revealed or actually studied in a wide variety of cases, which are of special interest to bacteriologists. Not less suggestive are the determinations of the true reaction of various natural solutions in which bacteria find their habitat.

The study of the disinfecting power of the hydrogen ions may be said to have begun with the work of Paul and Krönig (1896) and Krönig and Paul (1897) on bacteria, Clark's (1899) work with fungi and Bial's (1902) work with yeasts. These investigations, like many which were to follow, were handicapped by the lack of a method for the direct determination of hydrogen ion concentration. As late as 1906 Winslow and Lochridge, in their demonstration of the disinfecting power of hydrogen ions, had to resort to conductivity data, which are difficult to interpret when the solutions are complex. Even later, when better methods became available, elaborate researches were recorded in which the attempt was made to draw refined conclusions by comparing the effects of different acids with their dissociation constants. This procedure in other lines of investigation has yielded results which are far less certain than those obtained by direct determinations of hydrogen ion concentration, and for reasons which will shortly be mentioned. It may be well, therefore, to consider as unsettled some of the mooted points which have arisen in the study of the disinfecting power of the

hydrogen ions until some of the work which has been done even within the last year can be repeated with more modern methods of attack. It should be noted that some of the work upon this subject has indicated a not surprising specificity in the effects of different acids. Nevertheless we fail to see how these important differences can be accurately measured until a method is used which permits the determination of at least one of the products of dissociation.

Of particular interest is the work of Bruenn (1915) who found the disinfecting power of lactic and acetic acids for *B. coli* and *B. typhi* to be due to the activity of the hydrogen ions and who therefore opposed the conclusions of Paul, Birstein and Reuss (1910).

A fundamental objection which may be raised against some of the work which has been done in trying to discover the effects of acids or of the hydrogen ions is that no account has been taken of the "buffer action" in the media used. It is not a difficult matter to demonstrate theoretically and experimentally that in the presence of proper buffers acids may not furnish concentrations of the hydrogen ion which can be judged merely from an inspection of their dissociation constants. In many instances a "weak" acid may alter the hydrogen ion concentration to practically the same extent as the same quantity of a much "stronger" acid.

Unbuffered solutions, on the other hand, are susceptible to enormous changes in hydrogen ion concentration under the action of even certain of the so-called "neutral salts." Quite apart from their well-established influence upon the apparent hydrogen ion concentration of buffered solutions, such so-called neutral salts may at times exert a greater physiological influence by altering the hydrogen ion concentration of a solution than they do through the direct influence of their own ions.

These brief citations and remarks will be recognized by those who are familiar with the subject as a very inadequate sketch. They may, however, serve to indicate the basis upon which Sörensen (1912) stood when he maintained that in the study of biological processes no conclusion may be regarded as free from the possibility of error unless the influence of the hydrogen ions

has been controlled or taken into consideration. If this is true in the fields of bacteriological research, some experimental method of determining hydrogen ion concentrations which is particularly adapted to bacteriological problems is an absolute necessity.

Each of the two chief methods of determining hydrogen ion concentrations has its own merit. For accurate determinations, for the study of highly colored solutions, as a check upon colorimetric measurements and in all cases of serious importance the hydrogen electrode method should be available. It has been developed so that very high accuracy may generally be attained in less time than is required for very accurate colorimetric work. We must qualify this statement by adding; that for accurate, rapid work there is necessary temperature control, careful electrical installation, the use of a proper electrode vessel and the shaking method of establishing equilibria, attention to numerous details, and the use of a potentiometer system which is reliable.[2] Reviews of the electrometric method will be found in the monographs of Sörensen (1912) and Michaelis (1914 b). A hydrogen electrode vessel particularly adapted to accurate rapid work has been described by Clark (1915 b) and used in several bacteriological investigations. Such an equipment can now be purchased from a reliable manufacturer of electrical instruments.

There are, however, cases where the hydrogen electrode is not applicable, though we have reason to believe that these cases will become fewer as the theory and technique of electrometric measurements are perfected. There are also cases where

[2] McClendon (1915) and Bovie (1915) have described potentiometers designed to indicate directly the P_H value of a measured solution. Doubtless such instruments have special uses, but, for general work, we fail to see any real advantage in a "direct reading potentiometer." It seems almost unnecessary to call attention to the fact that, when rough measurements are made and corrections do not have to be applied, potential readings may be transformed to P_H in five seconds by means of a curve. If accurate measurements are to be made corrections must be applied, which is difficult with a direct reading potentiometer. If very rapid changes are to be followed, it is quite as easy to think of these in terms of potential as in terms of P_H. If direct readings must be had and corrections are to be neglected, a P_H scale pasted over the millivolt scale in Hildebrand's (1913) arrangement will do. We are convinced that it is well to use only a reliable universal instrument.

great accuracy is not necessary and a rapid, simple method will do. There are, furthermore, cases where it is necessary to make a great many approximate determinations of hydrogen ion concentrations, which need be of only *relatively* high accuracy. In such cases, which occur daily in the bacteriological laboratory, the colorimetric method finds its greatest usefulness.

This method has long been available, but, probably because it has not been developed to meet the particular needs of bacteriology, and also because its usefulness to the bacteriologist has not been sufficiently emphasized, it has found no extensive application in the bacteriological laboratory.

We have felt that one hindrance has been the lack of brilliant indicators which can reveal their complexion even in the presence of the coloration and the turbidity found in many culture media and cultures. This hindrance we have partially removed by assembling a series of exceptionally brilliant indicators, several of which we ourselves have synthesized for the first time. We have also studied some methods which materially aid in the examination of highly colored or turbid media, and at the same time we have developed a system of standard solutions and have simplified their preparation, so that, with regard to the several minor points which will be described, we feel that the method is readily available to the bacteriologist for routine as well as research purposes. The relative accuracy of the method will be revealed in the numerous comparisons we have made with careful electrometric determinations on a wide variety of solutions.

In order that the main outlines of the subject may not be lost in the discussion of experimental details, and in order that they may be assembled for the convenience of those bacteriologists who may desire a review of the subject, the more essential theoretical aspects will be briefly reviewed,[3] and their particular significance in bacteriological problems will be noted.

[3] An excellent elementary exposition of the theory of electrolytic dissociation is found in a little book by Talbot and Blanchard (1905). Fuller treatments will be found in any modern text book of physical chemistry. Those portions in which we are now especially interested have been treated in the monographs of Sörensen (1912) and Michaelis (1914).

Those already familiar with the subject will find the new information they desire in the following sections: A new series of standard comparison solutions is described in section V and the compositions and corresponding P_H values are given in table 1. A complete new series of indicators is listed in table 2. Comparisons of electrometric P_H determinations and colorimetric determinations made with these indicators are shown and briefly described in section XIV. A description of apparatus will be found in section XII.

SECTION II. THE METHOD OF EXPRESSING HYDROGEN ION CONCENTRATIONS

In physiological solutions there are generally encountered concentrations of the hydrogen ion which are very much smaller than the concentrations of the substances ordinarily dealt with in analytical chemistry. Nevertheless the standard of concentration remains the normal solution, which, by definition, contains the equivalent of 1 gram of hydrogen in 1 liter of the solution.[4] In these terms the hydrogen ion concentration of pure water, for instance, is 0.000,000,1 N. A convenient abbreviation of this unwieldy figure is 1×10^{-7}N. In like manner the hydrogen ion concentration of a 0.1 N NaOH solution, which is about 0.000,000,000,000,09 N, may be written 9×10^{-14}.

If the hydrogen ion concentration of pure water, which is considered to be that of *true neutrality*, is 1×10^{-7} N, it may be asked how one may speak of the hydrogen ion concentration of an *alkaline solution* and of a value as small as 9×10^{-14}. It follows from the fact that in every aqueous solution there are both hydrogen and hydroxyl ions, the relative concentrations of which are governed by the dissociation of the water itself. In the reversible reaction $H_2O \rightleftarrows \overset{+}{H} + \overline{O}H$, which expresses the dissociation of water, there is an equilibrium which may be expressed as follows:

[4] For all ordinary purposes it makes no difference in defining the normality of hydrogen ion concentrations whether we consider the atomic weight of hydrogen to be 1.0 or 1.008.

$$[H] \times [OH] = K_w$$

[H] and [OH] represent the concentrations of hydrogen and hydroxyl ions respectively, and K_w is a constant, the so-called dissociation constant of water. If K_w in the above equation is to remain a constant as [H] and [OH] change, there must be present in every aqueous solution some hydrogen ions and there must be maintained a balance between the concentrations [H] and [OH]. When by the addition of alkali, for instance, [OH] is increased there must remain sufficient hydrogen ions to make the product [H] × [OH] equal to the constant K_w.

K_w has been established by various methods with remarkable agreement. Although it varies with the temperature it may be considered here to be equal to 1×10^{-14}. Assuming, as we must, that in perfectly pure water the concentrations of the hydrogen and hydroxyl ions are equal we have:

$$[H]^2 = [OH]^2 = 1 \times 10^{-14}$$

Hence:— $$[H] = [OH] = 1 \times 10^{-7}$$

This concentration of hydrogen or hydroxyl ions (1×10^{-7}) is called *the true neutral point.* Solutions with hydrogen ion concentrations greater than or hydroxyl ion concentrations less than 1×10^{-7} are called acid solutions. Those with hydrogen ion concentrations less than or hydroxyl ion concentrations greater than 1×10^{-7} are called alkaline solutions. Since $[OH] = \frac{K_w}{[H]}$ the hydroxyl ion concentration of a solution may be readily calculated if its hydrogen ion concentration is known, or *vice versa.* As a matter of practice it is more convenient to determine hydrogen ion concentrations, and as a matter of uniformity only the hydrogen ion concentration of a solution is generally mentioned as was suggested by Friedenthal (1904).

It is often necessary to plot hydrogen ion concentrations upon coördinate paper. In this case a difficulty arises when one attempts to lay off on a chart concentrations which are widely different. If one wishes, for instance to show a difference between 1×10^{-5} and 3×10^{-3} the chart would not be within bounds when one came to the magnitudes 1×10^{-4} and 3×10^{-4}. We could, however, plot these values logarithmically as Hender-

son (1908) did in plotting dissociation curves. It has been very generally agreed that the logarithm of the reciprocal of the hydrogen ion concentration should be used. To this Sörensen (1909 a and b) has given the symbol P_H^+ which we shall write P_H. This particular function of the hydrogen ion concentration is especially convenient for the following reason among others. In the determination of hydrogen ion concentrations by the electrometric method the potentials measured are introduced into an equation which contains the term $\log. \frac{1}{[H].}$ The value of this term is obtained very directly and serves to characterize a hydrogen ion concentration quite as well as if the calculation were carried further to obtain the actual value of [H].

The relation of P_H to [H] may be shown by the following examples. Since $P_H = \log. \frac{1}{[H],}$

if $[H] = 2 \times 10^{-4}$, $P_H = \log. \frac{1}{2 \times 10^{-4}} = \log. \quad 5{,}000 = 3.699$

and

if $[H] = 4 \times 10^{-6}$, $P_H = \log. \frac{1}{4 \times 10^{-6}} = \log. 250{,}000 = 5.398$

The following section of a table showing the relation of P_H to [H] will illustrate the construction of the whole.

P_H	[H]
5.0	1.0×10^{-5}
5.1	7.9×10^{-6}
5.2	6.3×10^{-6}
5.3	5.0×10^{-6}
5.4	4.0×10^{-6}
5.5	3.2×10^{-6}
5.6	2.5×10^{-6}
5.7	2.0×10^{-6}
5.8	1.6×10^{-6}
5.9	1.2×10^{-6}
6.0	1.0×10^{-6}
6.1	7.9×10^{-7}
6.2	6.3×10^{-7}

—etc.—

The use of Sörensen's P_H has met with superficial objections such as those set up by Armstrong and Armstrong (1913), and other scales of reaction have been proposed such as the very inadequate one suggested by Walker and Kay (1912). Nevertheless P_H will doubtless continue to be used, not only as a matter of custom but because it is a fundamentally logical unit. Our methods of determining hydrogen ion concentrations have errors which are more or less directly proportional to some constant difference of P_H but are not directly proportional to any constant difference in [H]. Differences in P_H rather than differences in [H] are of physiological importance, and finally the graphic treatment of the relation of hydrogen ion concentrations to the dissociation of acids and bases may be expressed most beautifully when P_H values rather than the corresponding [H] values are used as Henderson and Sörensen and others have shown. We shall therefore use the P_H values rather than the corresponding hydrogen ion concentrations, in the following discussions.

It should be particularly noted that as P_H increases [H] decreases and *vice versa*. In plotting curves where we wish to show the relation between P_H and some other quantity, such as the amount of acid added to a medium, we shall depart from the common practice of showing a rise in P_H by a rise in the curve, instead plotting P_H so that a rise in the curve at once indicates to the eye a rise in acidity.

SECTION III. BUFFER ACTION

If we were to add to 1 liter of perfectly pure water of $P_H = 7.0$, 1 cc. of 0.01 N HCl, the resulting solution would be about $P_H = 5.0$ and very toxic to many bacteria. If, on the other hand, we were to add this same amount of acid to a liter of a standard beef infusion medium of $P_H = 7.0$, the resulting change in P_H would be hardly appreciable. This power of certain solutions to resist change in reaction was commented upon by Fernbach and Hubert (1900) who likened the resistance of phosphate solutions to a "tampon." The word was adopted by Sörensen (1909 a) and in the German rendition of his paper (1909 b) it

became "puffer" and thence the English "buffer." There has been some objection to this word so applied, but it has now acquired a clear technical meaning and is so generally used that it should probably be retained. By buffer action then we mean the ability of a solution to resist change in P_H through the addition or loss of acid or alkali. This may be illustrated by titration curves such as those shown in figures 1, 2 and 3. The construction of such curves may be illustrated by the following example.

A 1 per cent solution of Witte peptone was found to have a P_H value of 6.87. To equal portions of the solution were added successively increasing amounts of 0.1 N lactic acid and the resulting P_H was measured in each case. There were also added to equal portions of the solution successively increasing amounts of 0.1 N NaOH and the resulting P_H was measured in each case. The P_H values were then plotted on cross section paper as ordinates against the amount of acid or alkali added in each case as abscissas. This gave the curve shown in figure 1. The other curve shown in this figure was constructed with data obtained with a 5 per cent solution of Witte peptone. The curves of figures 2 and 3 were obtained in a similar way.

These curves illustrate the following points. Figure 1 shows that the buffer action of a solution is dependent upon the concentration of the constituents. The 5 per cent solution is much more resistant to change in P_H than the 1 per cent. solution. It will also be noticed that in either case the buffer action is not the same at all points in the curve. In other words the buffer action can not be expressed by a constant but must be determined for each region of P_H. This is illustrated even more clearly by the titration curve for phosphoric acid, (fig. 2). At the point where the solution contains only the primary phosphate, and again where it contains only the secondary phosphate there is very little buffer effect indeed. Furthermore the buffer action of a solution may not be due entirely to the nature of the constituents titrated but also to the nature of the substance with which it is titrated. This point may be illustrated by titrating a beef infusion medium in the one case with hydro-

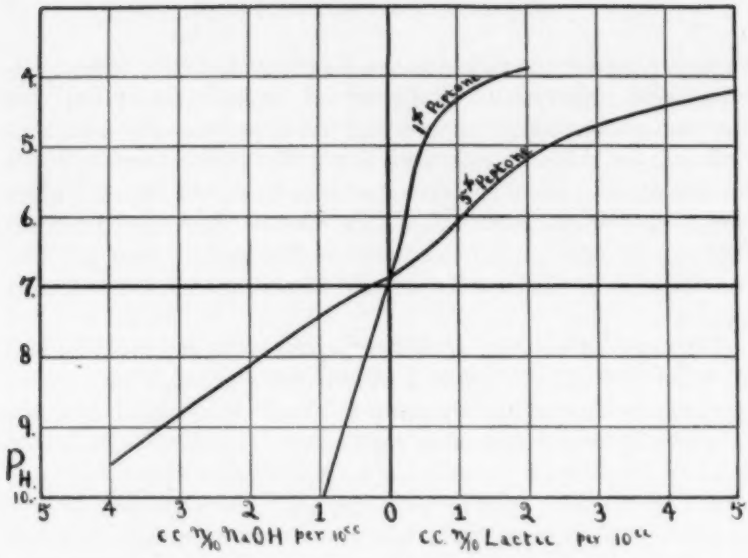

FIG. 1. TITRATION CURVES OF PEPTONE SOLUTIONS

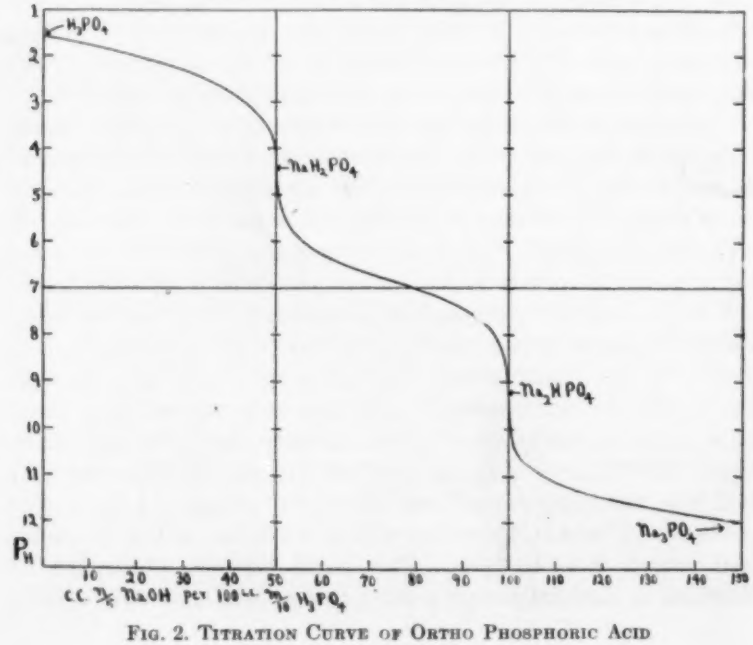

FIG. 2. TITRATION CURVE OF ORTHO PHOSPHORIC ACID

chloric acid and in the other case with acetic acid both of the same normality (see fig. 3). It will be seen that at first the two curves are identical. As the region is approached where the dissociation of the "weak" acetic acid is itself suppressed because of the accumulation of acetate ions and the high concentration of the hydrogen ions, further addition of this acid has com-

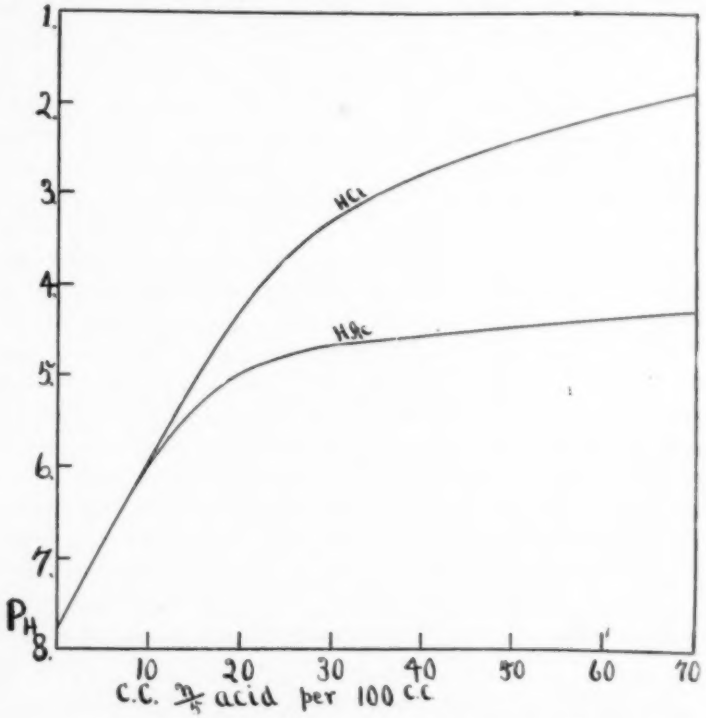

FIG. 3. TITRATION CURVES OF A BEEF INFUSION MEDIUM

paratively little effect. The strongly dissociated hydrochloric acid, on the other hand continues to be effective until it too, at very high hydrogen ion concentrations, is suppressed.

These examples will suffice to make it evident that the buffer action of a solution is dependent upon the nature and the concentrations of the constituents, upon the P_H region where the

buffer action is measured, and upon the nature of the acid or alkali added. To connect all these variables is a difficult problem. Koppel and Spiro (1914) have attempted to do so, but they have necessarily had to leave out of consideration another factor. If there are present any bodies which tend to absorb any of the constituents of a solution which can affect the hydrogen ion concentration of a solution, these bodies will tend to act as buffers, or will affect the buffer action of the solution. Henderson (1909) has called attention to this and Bovie (1915) has shown in a very interesting way the buffer action of charcoal. Since some culture media or cultures, and many of the solutions whose buffer action must be studied for physiological purposes, contain undissolved or colloidal material which may act in this way, it seems best to consider buffer action in its broadest sense, and to express it by the relative slopes of titration curves determined experimentally. Further illustrations of titration curves of culture media will be found in the papers of Clark (1915b and e) and of Bovie (1915). Titration curves of some inorganic solutions will be found in a paper by Hildebrand (1913). More theoretical treatments of the subject are given in the papers of Henderson (1909), Sörensen (1909 a and b), Sörensen (1912,) Michaelis (1914 b) and Koppel and Spiro (1914).

The standard comparison solutions used in the colorimetric determination of P_H are buffer solutions. Only those solutions may be used for standards which have a buffer action sufficient to resist the effect of unavoidable slight contamination. The curves for some standard solutions are shown in figure 4.

SECTION IV. OUTLINE OF THE COLORIMETRIC METHOD

The colorimetric method of determining hydrogen ion concentrations is based upon the fact that each indicator has a characteristic zone of hydrogen ion concentrations within which its color changes occur. Thus phenolphthalein is colorless above $[H] = 1 \times 10^{-8}$ ($P_H = 8$), but, if the solution is slightly more alkaline the pink color appears and becomes more intense with increased alkalinity until the indicator seems to have its full

color at about $P_H = 10$. Methyl red, which is yellow in alkaline, neutral and slightly acid solutions, and red in very acid solutions, exhibits its intermediate colors between $P_H = 4$ and $P_H = 6$.

If certain physical constants of indicators were well enough established, and, if we had convenient means of accurately and rapidly determining the percentage color change induced, we could use such data to determine hydrogen ion concentrations; and such a method could be made in large measure independent of all others.

It is much more convenient however to have a set of buffer solutions whose P_H values have been accurately defined by hydrogen electrode measurements, and to compare their color after the addition of the proper indicator with the color of the tested solution to which a like quantity of the indicator has been added.

The essentials then are: first a set of indicators to cover the ranges of P_H to be studied, and second a set of standard buffer solutions which may be used within these ranges.

SECTION V. STANDARD SOLUTIONS

The standard solutions used in the colorimetric method of determining hydrogen ion concentrations are buffer solutions with such well defined compositions that they can be accurately reproduced, and with P_H values accurately defined by hydrogen electrode measurements. They generally consist of mixtures of some acid and its alkali salt. Several such mixtures have been carefully studied. An excellent set has been described by Sörensen (1912). This set may be supplemented by the acetic acid—sodium acetate mixtures, most careful measurements of which have been made by Walpole (1914 b), and by Palitzsch's (1915) excellent boric acid-borax mixtures. In a recent paper (Clark and Lubs, 1916) we have described a set of standards which we believe is well designed for ease, convenience and accuracy of preparation. We called attention to certain slight difficulties in the preparation of the older standards, which we believe

should be avoided if the colorimetric method is to come into the wide routine use which we hope it will enjoy in bacteriological studies; and we noted the advantages of the following set of mixtures:

Potassium chlorid + HCl
Acid potassium phthalate + HCl
Acid potassium phthalate + NaOH
Acid potassium phosphate + NaOH
Boric acid, KCl + NaOH

For a discussion of these mixtures, the methods used in determining their P_H values, and the potential measurements we refer the reader to the original paper [Journal of Biological Chemistry, 1916, vol. xxv, no. 3, p. 479]. We may proceed at once to describe the details of preparation.

The various mixtures are made up from the following stock solutions: M/5 potassium chlorid (KCl), M/5 acid potassium phosphate (KH_2PO_4), M/5 acid potassium phthalate ($KHC_8H_4O_4$), M/5 boric acid with M/5 potassium chlorid (H_3BO_3, KCl), M/5 sodium hydroxid (NaOH), and M/5 hydrochloric acid (HCl). Although the subsequent mixtures are diluted to M/20 the above concentrations of the stock solutions are convenient for several reasons.

The water used in the crystallization of the salts and in the preparation of the stock solutions and mixtures should be redistilled. So-called "conductivity water," which is distilled first from acid chromate solution and again from barium hydroxid, is recommended.

M/5 potassium chlorid solution. (This solution will not be necessary except in the preparation of the most acid series of mixtures.) The salt should be recrystallized three or four times and dried in an oven at about 120°C. for two days. The fifth molecular solution contains 14.912 grams in 1 liter.

M/5 Acid potassium phthalate solution. Acid potassium phthalate may be prepared by the method of Dodge (1915) modified as follows. Make up a concentrated potassium hydroxid solution by dissolving about 60 grams of a high grade sample in about 400 cc. of water. To this add 50 grams of the commercial

resublimed anhydrid of ortho phthalic acid. Test a cool portion of the solution with phenol phthalein. If the solution is still alkaline, add more phthalic anhydrid; if acid, add more KOH. When roughly adjusted to a slight pink with phenol phthalein[5] add as much more phthalic anhydrid as the solution contains and heat till all is dissolved. Filter while hot, and allow the crystallization to take place slowly. The crystals should be drained with suction and recrystallized at least twice from distilled water.[6] Dry the salt at 110°–115°C. to constant weight.

A fifth molecular solution contains 40.828 grams of the salt in 1 liter of the solution.

M/5 acid potassium phosphate solution. A high grade commercial sample of the salt is recrystallized at least three times from distilled water and dried to constant weight at 110°–115°C. A fifth molecular solution should contain in 1 liter 27.232 grams. The solution should be distinctly red with methyl red and distinctly blue with brom phenol blue.

M/5 boric acid M/5 potassium chloride. Boric acid should be recrystallized several times from distilled water. It should be air dried[7] in thin layers between filter paper and the constancy of weight established by drying small samples in thin layers in a desiccator over $CaCl_2$. Purification of KCl has already been noted. It is added to the boric acid solution to bring the salt concentration in the borate mixtures to a point comparable with that of the phosphate mixtures so that colorimetric checks may be obtained with the two series where they overlap. One liter of the solution should contain 12.4048 grams of boric acid and 14.912 grams of potassium chlorid.

M/5 Sodium hydroxid solution. This solution is the most difficult to prepare, since it should be as free as possible from carbonate. A solution of sufficient purity for the present purposes may be prepared from a high grade sample of the hydroxid

[5] Use a diluted portion for the final test.

[6] While the present war-price of phthalic acid continues it will be well to recover the phthalic acid from the mother liquors by acidifying these. The recovered phthalic acid may be easily and economically purified by several recrystallizations.

[7] Boric acid begins to lose "water of constitution" above 50°C.

in the following manner. Dissolve 100 grams NaOH in 100 cc. distilled water in a Jena or Pyrex glass Erlenmeyer flask. Cover the mouth of the flask with tin foil and allow the solution to stand over night till the carbonate has mostly settled. Then prepare a filter as follows. Cut a "hardened" filter paper to fit a Buchner funnel. Treat it with warm strong [1 : 1] NaOH solution. After a few minutes decant the sodium hydroxid and wash the paper first with absolute alcohol, then with dilute alcohol, and finally with large quantities of distilled water. Place the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated but do not dry so that the paper curls. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod making sure that the paper, under gentle suction, adheres well to the funnel, and draw the solution through with suction. The clear filtrate is now diluted quickly, after rough calculation, to a solution somewhat more concentrated than N/1. Withdraw 10 cc. of this dilution and standardize roughly with an acid solution of known strength, or with a sample of acid potassium phthalate. From this approximate standardization calculate the dilution required to furnish an M/5 solution. Make the required dilution with the least possible exposure, and pour the solution into a *paraffined* bottle to which a calibrated 50 cc. burette and soda-lime guard tubes have been attached. See section XII. The solution should now be most carefully standardized. One of the simplest methods of doing this, and one which should always be used in this instance, is the method of Dodge (1915) in which use is made of the acid potassium phthalate purified as already described. Weigh out accurately on a chemical balance with standardized weights several portions of the salt of about 1.6 grams each. Dissolve in about 20 cc. distilled water and add 4 drops phenol phthalein. Pass a stream of CO_2—free air through the solution and titrate with the alkali till a faint but distinct and permanent pink is developed. It is preferable to use a factor with the solution rather than attempt adjustment to an exact M/5 solution.

M/5 Hydrochloric acid solution. Dilute a high grade of hydro-

chloric acid solution to about 20 per cent and distill. Dilute the distillate to approximately M/5 and standardize with the sodium hydroxid solution previously described. If convenient, it is well to standardize this solution carefully by the silver chlorid method and check with the standardized alkali.

The only solution which it is absolutely necessary to protect from the CO_2 of the atmosphere is the sodium hydroxid solution. Therefore all but this solution may be stored in ordinary bottles of resistant glass. The salt solutions, if adjusted to exactly M/5, may be measured from clean calibrated pipettes.

These constitute the stock solutions from which the mixtures are prepared. The general relationships of these mixtures to their P_H values are shown in figure 4. In this figure P_H values are plotted as ordinates against X cc. of acid or alkali as abscissae. It will be found convenient to plot this figure from the following table with greatly enlarged scale so that it may be used as is Sörensen's chart (1909 a). The composition of the mixtures at even intervals of 0.2 P_H are given in table 1.

In any measurement the apportionment of scale divisions should accord with the precision. Scale divisions should not be so coarse that interpolations tax the judgment nor so fine as to be ridiculous. What scale divisions are best in the method under discussion it is difficult to decide, since the precision which may be attained depends somewhat upon the ability of the individual eye, and upon the material examined, as well as upon the means and the judgment used in overcoming certain difficulties which we shall mention later. Certain general considerations have led us to believe that for most bacteriological work estimation of P_H values to the nearest 0.1 division is sufficiently precise, and that this precision can be obtained when the composition of the medium permits if the comparison standards differ by increments of 0.2 P_H. Sörensen (1909 a and b) has arranged the standard solutions to differ by even parts of the components, a system which furnishes uneven increments in P_H. Michaelis, (1910) on the other hand, makes his standards vary by about 0.3 P_H so that the corresponding hydrogen ion

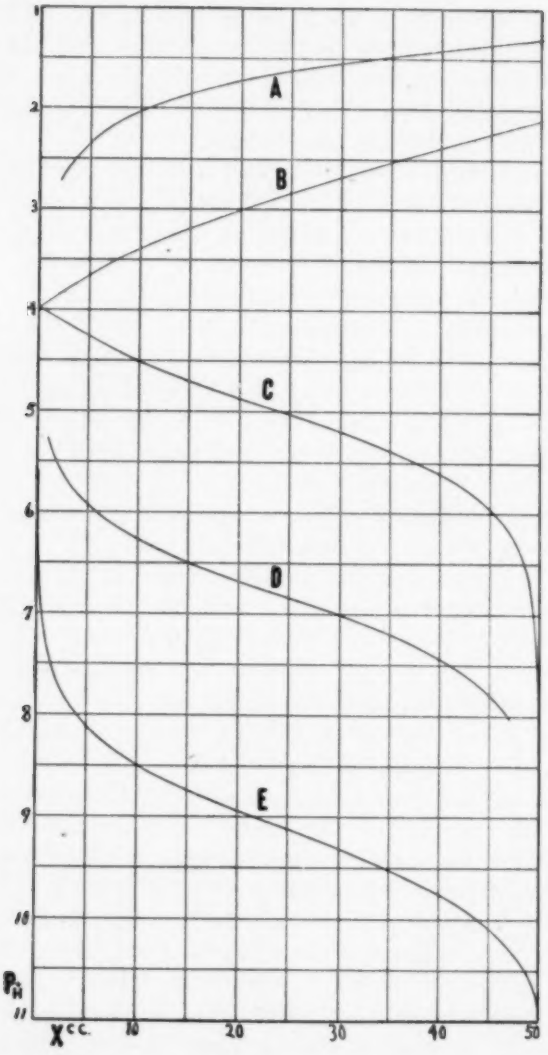

FIG. 4. P_H VALUES OF THE MIXTURES:

A.	50 cc. 0.2 M KCl	+ X cc. 0.2 M HCl	Diluted to 200
B.	50 cc. 0.2 M HKPhthalate	+ X cc. 0.2 M HCl	Diluted to 200
C.	50 cc. 0.2 M HKPhthalate	+ X cc. 0.2 M NaOH	Diluted to 200
D.	50 cc. 0.2 M H_2KPO_4	+ X cc. 0.2 M NaOH	Diluted to 200
E.	50 cc. 0.2 M H_3BO_3, 0.2 M KCl	+ X cc. 0.2 M NaOH	Diluted to 200

concentrations are approximately doubled at each step. Our experience has convinced us of the advantage of the 0.2 P_H increments we are recommending.

We have found it convenient to prepare 200 cc. of each of the mixtures and to preserve them in bottles each of which has its own 10 cc. pipette thrust through the stopper. It takes but little more time to prepare 200 cc. than it does to prepare a 10

TABLE 1

Compositions of mixtures giving P_H values at 20°C. at intervals of 0.2

P_H	*Composition*		
KCl-HCl mixtures			
1.0	50 cc. M/5 KCl	97.0 cc. M/5 HCl	Dilute to 200 cc.
1.2	50 cc. M/5 KCl	64.5 cc. M/5 HCl	Dilute to 200 cc.
1.4	50 cc. M/5 KCl	41.5 cc. M/5 HCl	Dilute to 200 cc.
1.6	50 cc. M/5 KCl	26.3 cc. M/5 HCl	Dilute to 200 cc.
1.8	50 cc. M/5 KCl	16.6 cc. M/5 HCl	Dilute to 200 cc.
2.0	50 cc. M/5 KCl	10.6 cc. M/5 HCl	Dilute to 200 cc.
2.2	50 cc. M/5 KCl	6.7 cc. M/5 HCl	Dilute to 200 cc.
Phthalate-HCl mixtures			
2.2	50 cc. M/5 KHPhthalate	46.70 cc. M/5 HCl	Dilute to 200 cc.
2.4	50 cc. M/5 KHPhthalate	39.60 cc. M/5 HCl	Dilute to 200 cc.
2.6	50 cc. M/5 KHPhthalate	32.95 cc. M/5 HCl	Dilute to 200 cc.
2.8	50 cc. M/5 KHPhthalate	26.42 cc. M/5 HCl	Dilute to 200 cc.
3.0	50 cc. M/5 KHPhthalate	20.32 cc. M/5 HCl	Dilute to 200 cc.
3.2	50 cc. M/5 KHPhthalate	14.70 cc. M/5 HCl	Dilute to 200 cc.
3.4	50 cc. M/5 KHPhthalate	9.90 cc. M/5 HCl	Dilute to 200 cc.
3.6	50 cc. M/5 KHPhthalate	5.97 cc. M/5 HCl	Dilute to 200 cc.
3.8	50 cc. M/5 KHPhthalate	2.63 cc. M/5 HCl	Dilute to 200 cc.
Phthalate-NaOH mixtures			
4.0	50 cc. M/5 KHPhthalate	0.40 cc. M/5 NaOH	Dilute to 200 cc.
4.2	50 cc. M/5 KHPhthalate	3.70 cc. M/5 NaOH	Dilute to 200 cc.
4.4	50 cc. M/5 KHPhthalate	7.50 cc. M/5 NaOH	Dilute to 200 cc.
4.6	50 cc. M/5 KHPhthalate	12.15 cc. M/5 NaOH	Dilute to 200 cc.
4.8	50 cc. M/5 KHPhthalate	17.70 cc. M/5 NaOH	Dilute to 200 cc.
5.0	50 cc. M/5 KHPhthalate	23.85 cc. M/5 NaOH	Dilute to 200 cc.
5.2	50 cc. M/5 KHPhthalate	29.95 cc. M/5 NaOH	Dilute to 200 cc.
5.4	50 cc. M/5 KHPhthalate	35.45 cc. M/5 NaOH	Dilute to 200 cc.
5.6	50 cc. M/5 KHPhthalate	39.85 cc. M/5 NaOH	Dilute to 200 cc.
5.8	50 cc. M/5 KHPhthalate	43.00 cc. M/5 NaOH	Dilute to 200 cc.
6.0	50 cc. M/5 KHPhthalate	45.45 cc. M/5 NaOH	Dilute to 200 cc.
6.2	50 cc. M/5 KHPhthalate	47.00 cc. M/5 NaOH	Dilute to 200 cc.

TABLE 1—*Continued*

KH_2PO_4-NaOH mixtures

5.8	50 cc. M/5 KH_2PO_4	3.72 cc. M/5 NaOH	Dilute to 200 cc.
6.0	50 cc. M/5 KH_2PO_4	5.70 cc. M/5 NaOH	Dilute to 200 cc.
6.2	50 cc. M/5 KH_2PO_4	8.60 cc. M/5 NaOH	Dilute to 200 cc.
6.4	50 cc. M/5 KH_2PO_4	12.60 cc. M/5 NaOH	Dilute to 200 cc.
6.6	50 cc. M/5 KH_2PO_4	17.80 cc. M/5 NaOH	Dilute to 200 cc.
6.8	50 cc. M/5 KH_2PO_4	23.65 cc. M/5 NaOH	Dilute to 200 cc.
7.0	50 cc. M/5 KH_2PO_4	29.63 cc. M/5 NaOH	Dilute to 200 cc.
7.2	50 cc. M/5 KH_2PO_4	35.00 cc. M/5 NaOH	Dilute to 200 cc.
7.4	50 cc. M/5 KH_2PO_4	39.50 cc. M/5 NaOH	Dilute to 200 cc.
7.6	50 cc. M/5 KH_2PO_4	42.80 cc. M/5 NaOH	Dilute to 200 cc.
7.8	50 cc. M/5 KH_2PO_4	45.20 cc. M/5 NaOH	Dilute to 200 cc.
8.0	50 cc. M/5 KH_2PO_4	46.80 cc. M/5 NaOH	Dilute to 200 cc.

Boric acid, KCl-NaOH mixtures

7.8	50 cc. M/5 H_3BO_3, M/5 KCl	2.61 cc. M/5 NaOH	Dilute to 200 cc.
8.0	50 cc. M/5 H_3BO_3, M/5 KCl	3.97 cc. M/5 NaOH	Dilute to 200 cc.
8.2	50 cc. M/5 H_3BO_3, M/5 KCl	5.90 cc. M/5 NaOH	Dilute to 200 cc.
8.4	50 cc. M/5 H_3BO_3, M/5 KCl	8.50 cc. M/5 NaOH	Dilute to 200 cc.
8.6	50 cc. M/5 H_3BO_3, M/5 KCl	12.00 cc. M/5 NaOH	Dilute to 200 cc.
8.8	50 cc. M/5 H_3BO_3, M/5 KCl	16.30 cc. M/5 NaOH	Dilute to 200 cc.
9.0	50 cc. M/5 H_3BO_3, M/5 KCl	21.30 cc. M/5 NaOH	Dilute to 200 cc.
9.2	50 cc. M/5 H_3BO_3, M/5 KCl	26.70 cc. M/5 NaOH	Dilute to 200 cc.
9.4	50 cc. M/5 H_3BO_3, M/5 KCl	32.00 cc. M/5 NaOH	Dilute to 200 cc.
9.6	50 cc. M/5 H_3BO_3, M/5 KCl	36.85 cc. M/5 NaOH	Dilute to 200 cc.
9.8	50 cc. M/5 H_3BO_3, M/5 KCl	40.80 cc. M/5 NaOH	Dilute to 200 cc.
10.0	50 cc. M/5 H_3BO_3, M/5 KCl	43.90 cc. M/5 NaOH	Dilute to 200 cc.

cc. portion, and if the larger volume is prepared there will not only be a sufficient quantity for a day's work but there will be some on hand for the occasional test.

Unless electrometric measurements can be used as control, we urge the most scrupulous care in the preparation and preservation of the standards. Mixtures over a week old should be discarded. We believe that the use of preservatives, such as toluol, is likely to lead to a false sense of security and we have therefore not attempted to investigate their use. We have specified several recrystallizations of the salts used because no commercial samples which we have yet encountered are reliable. Dependence on these commercial salts has caused errors in the preparation of the old standards in important cases.

It is important to check the consistency of any particular set of these mixtures by comparing "5.8" and "6.2 phthalate" with "5.8" and "6.2 phosphate" using brom cresol purple. Also "7.8" and "8.0 phosphate" should be compared with the corresponding borates using cresol red.

SECTION VI. CHOICE OF INDICATORS

There is an almost unlimited number of compounds which have indicator properties. Among those of plant origin litmus and alizarine and among those of animal origin cochineal are the most familiar. An enormous number of plant pigments have been described, and the indicator properties of several have been noted. These have generally been described merely as indicators with no data of any value. Noteworthy exceptions have been Walpole's (1913) treatment of litmus and Walbum's (1913 a) study of the coloring principle of the red cabbage. As Walbum (1913 b) has pointed out, the mere description of a substance as an indicator is of no value whatever for biochemical purposes until its conduct in the presence of the substances found in biochemical solutions has been studied.

The indicators of natural origin deserve more study not only because of their possible general usefulness but because in certain instances, if they can be shown to exist in true solution, they may be made to furnish indications of the true reactions of the plasma in which they occur. They will then be useful in studies such as those attempted by Harvey (1913, 1915) and by Crozier (1916) upon the penetration of cells by acids and bases. In other instances their indications of the true reaction of plant tissues or juices may be of direct value to the plant pathologist.[8] Some of the pigments of bacterial origin are also worthy of investigation from the indicator point of view. Aside from the intrinsic interest of such studies they might reveal the possi-

[8] Some abortive attempts to connect the immunity of certain plant tissues with their "titratable acidities" might have been fruitful had the hydrogen ion concentrations been studied. Wagner's (1915) study of the hydrogen ion concentrations of infected plant "cell sap" seems to be a step in this direction.

bility of distinguishing these pigments by their characteristic transformation points.

Litmus and azolitmin have rendered valuable service in the survey work of bacteriology and may be given special notice. Ordinary litmus is a complex (Glazer, 1901), the composition and sensitivity of which will vary with the nine or more different methods of purification and with the source. The azolitmin of commerce, which is probably a constituent of litmus (Glazer), is of uncertain composition (Scheitz, 1910). It is very generally recognized that both litmus and azolitmin are inferior indicators which merit mention in only a few of the modern treatises.

With the development of organic chemistry many of the plant pigments have become products of the laboratory, and among the vast number of new compounds which have been synthesized are many which have admirable indicator properties. These synthetic indicators have for the most part displaced the natural indicators so that litmus and alizarine, turmeric and cochineal are relatively unfamiliar substances in the modern chemical laboratory. Indeed Bjerrum (1915) has stated that for most titrimetric purposes the two synthetic indicators methyl red and phenol phthalein are all that are necessary.

On the other hand, several indicators are required for the colorimetric determination of hydrogen ion concentrations. After Salessky (1904) in Nernst's laboratory had determined the hydrogen ion concentrations at which several indicators change, Friedenthal, and Fels, and Salm at about the same time (1904) placed the use of indicators upon a more comprehensive basis. It remained however for Sörensen (1909 a and b) not only to make the method practical and accurate in many details, but to reject the less useful indicators and to furnish a reliable selection. Indeed not the least of Sörensen's contributions has been the laborious elimination of inferior indicators. We need now no longer be discouraged by the bewildering choice presented in the elaborate indicator charts such as the classic one of Salm (1906) or the later one of Thiel (1911). Emphasis may be placed upon this part of Sörensen's work because the inferior indicators are still being used by many

unaware of their defects. We are surprised to find the reliance which Crile (1915) and Crozier, Rogers and Harrison (1915) have placed upon alizarine sulfonate. Walpole (1914 a) says "this indicator merits no place in any list of approved indicators for H concentration measurement." This is a strong statement which we believe the Cleveland authors should meet by more data than they have given, especially when we consider the profound importance of the phenomena which they have studied with this indicator.

Sörensen's selection was based largely upon the protein and salt errors which limit the usefulness of practically every indicator when applied to biological fluids. Those indicators chosen were characterized by *relatively* small susceptibility to the influence of protein and salts. To this list have since been added several other useful indicators. α-naphthol phthalein, first made by Grabowski (1871), was shown to be valuable by the studies of Sörensen and Palitzsch (1910). Methyl red [Rupp and Loose 1908] was given a permanent place by the investigations of Palitzsch (1911 b). Some indication of the usefulness of phenol sulfon phthalein [Sohon 1898] was suggested by the work of Levy, Rowntree and Marriot (1915). Several other indicators such as 2–5 di nitro hydro quinone, studied by Henderson and Forbes (1910), and the several indicators which were reviewed by Walpole (1914 a) may find uses. Hottinger (1914) has described an indicator which he isolated from lacmoid and which he calls "lacmosol," and Scatchard and Bogert (1916) have suggested that di nitro benzoylene urea is useful in biological solutions.

To these indicators which have been studied to determine their usefulness in the determination of hydrogen ion concentrations may be added the ever increasing list of indicators which have been described merely as indicators but whose usefulness remains to be determined. Dox's (1915) phenol quinolinein, the several indicators of the methyl red series which Rupp (1915) has again taken up, numerous derivatives of phenol phthalein, as well as many other indicators which are men-

tioned both in the old literature and the new, await a study of their usefulness.

Our own starting point was the selection of Sörensen (1909 a and b) supplemented by methyl red, α-napthol phthalein and phenol sulfon phthalein. Several of the Sörensen indicators were not available. We experimented with Henderson and Forbes' di nitro hydro quinone, Hottinger's lacmosol, several of the older synthetic indicators, Walbum's red cabbage extract, Sacher's (1910) red radish extract, Barthe's (1913) red potato extract, and several other natural indicators. Nierenstein (see Walpole) kindly sent us a sample of his indicator but we have been unable to make a careful study of it as yet.

The brilliancy of methyl red and of phenol sulfon phthalein led us to study other indicators of these two distinct types. Our preliminary report [Lubs and Clark 1915] will be found elsewhere.

In the methyl red series we studied the following:

o-carboxy benzene azo mono methyl aniline, Sive and Jones (1915),

o-carboxy benzene azo di methyl aniline, Rupp and Loose (1908).

o-carboxy benzene azo mono ethyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo di ethyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo mono propyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo di propyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo (?) amyl aniline[9] Lubs and Clark (1915).

[9] This is probably the mono alkyl compound. It was prepared by coupling di iso amyl aniline with diazotized anthranilic acid. By analogy with the preparation of di methyl red for instance, the di alkyl indicator should have been obtained. Karrer (1915), however, has shown that in the coupling of di butyl or di amyl aniline with a diazotized compound one alkyl group is split off. The weak color of the compound which we obtained indicates, by analogy with the weak color of the mono methyl, mono ethyl and mono propyl homologues, that it is the mono amyl compound instead of the di amyl compound. Limitations in the amount of materials available prevented adequate analysis to determine this.

o-carboxy benzene azo di methyl α naphthyl amine, Howard and Pope (1911).

o-carboxy benzene azo α naphthyl amine, Howard and Pope (1911).

o-carboxy benzene azo di phenyl amine, Howard and Pope (1911).

Meta carboxy benzene azo di methyl aniline[10] Lubs and Clark.

The mono alkyl homologues of methyl red were found to be much less brilliant than the di alkyl compounds and were therefore rejected. For the same reason or because of large protein errors we rejected the other compounds with the exception of di ethyl and di propyl red. Of these we have retained di propyl red because it is very useful in solutions of a little lower hydrogen ion concentration than those which may be studied with methyl red. Di propyl red which we shall henceforth refer to as propyl red, has some disadvantage because of its slight solubility, but this does not seriously interfere with its usefulness. Methyl red, o-carboxy benzene azo di methyl aniline, has already been extensively studied by others.

An indicator of the phthalein series which we should have mentioned in our first compilation is ortho cresol phthalein, first made by Fraude (1880). Its color change occurs in about the same range as that of phenol phthalein and is so much more brilliant that we believe ortho cresol phthalein can with advantage replace phenol phthalein for titrations.

Levy, Rowntree and Marriot first called attention to the usefulness of phenol sulfon phthalein for the determination of the hydrogen ion concentrations of dialized blood serum. This indicator, first made by Sohon (1898) in Remsen's laboratory, has received considerable attention from Acree and his students (see White 1915 and White and Acree, 1915) because it furnishes excellent material for the study of the quinone-phenolate theory of color change. New derivatives of phenol sulfon phthalein have been synthesized by Acree and his students for the purpose

[10] This indicator was prepared since the publication of our first report. It was prepared by coupling di methyl aniline with diazotized meta amino benzoic acid.

of furthering studies on tautomerism and the theory of color change in indicators, and new homologues and their derivatives have been synthesized by us in order to obtain satisfactory indicators for the determination of hydrogen ion concentration. Our preliminary work with these compounds has been briefly described in a former paper [Lubs and Clark 1915]. In this paper will be found those essential electrometric comparisons which are necessary before the usefulness of any indicator in biological solutions is established and which were neglected by Levy, Rowntree and Marriot (1915) and by Hurwitz, Meyer and Ostenberg (1915, 1916) in their studies of phenol sulfon phthalein.

In the sulfon phthalein series we studied the following:
Phenol sulfon phthalein, Sohon (1898).
Tetra nitro phenol sulfon phthalein, White and Acree (1915).
Phenol nitro sulfon phthalein, Lubs and Clark (1915).
Tetra bromo phenol sulfon phthalein, White and Acree (1915).
Tetra chloro phenol sulfon phthalein,[11] Lubs and Clark.
Ortho cresol sulfon phthalein, Sohon (1898).
Di bromo ortho cresol sulfon phthalein, Sohon (1898).
Thymol sulfon phthalein, Lubs and Clark (1915).
Thymol nitro sulfon phthalein,[11] Lubs and Clark.
Di bromo thymol sulfon phthalein, Lubs and Clark (1915).
α-napthol sulfon phthalein,[12] Lubs and Clark (1915).
Carvacrol sulfon phthalein,[11] Lubs and Clark.
Orcinol sulfon phthalein, Gilpin (1894).

Of these sulfon phthalein indicators we have chosen the following:—the phenol and tetra bromo phenol, the thymol and di bromo thymol and the o-cresol and di bromo o-cresol sulfon

[11] These indicators were prepared since the publication of our preliminary report on new indicators.

[12] Since the publication of our preliminary report we have received several inquiries concerning α-naphthol sulfon phthalein from those who desire an indicator useful in the alkaline region beyond the range covered by phenol sulfon phthalein. Although the α-naphthol compound may be useful, its solutions decompose so readily that we have entirely abandoned its use. The range within which it is useful is much more satisfactorily covered by other sulfon phthalein indicators.

phthaleins. These form a series useful between $P_H = 1.0$ and $P_H = 10.0$. With the exception of a portion of the range covered by methyl red this series is without serious gaps. A list of the useful ranges is given in table 2.

TABLE 2

List of indicators

CHEMICAL NAME	COMMON NAME	CONCENTRATION	COLOR CHANGE	RANGE P_H
		per cent		
Thymol sulfon phthalein (acid range)	Thymol blue	0.04	Red-yellow	1.2–2.8
Tetra bromo phenol sulfon phthalein	Brom phenol blue	0.04	Yellow-blue	3.0–4.6
Ortho carboxy benzene azo di methyl aniline	Methyl red	0.02	Red-yellow	4.4–6.0
Ortho carboxy benzene azo di propyl aniline	Propyl red	0.02	Red-yellow	4.8–6.4
Di bromo ortho cresol sulfon phthalein	Brom cresol purple	0.04	Yellow-purple	5.2–6.8
Di bromo thymol sulfon phthalein	Brom thymol blue	0.04	Yellow-blue	6.0–7.6
Phenol sulfon phthalein	Phenol red	0.02	Yellow-red	6.8–8.4
Ortho cresol sulfon phthalein	Cresol red	0.02	Yellow-red	7.2–8.8
Thymol sulfon phthalein (see above)	Thymol blue	0.04	Yellow-blue	8.0–9.6
Ortho cresol phthalein	Cresol phthalein	0.02	Colorless-red	8.2–9.8

With the improved method for the preparation of the sulfon phthalein indicators described by Lubs and Clark (1915) they may easily be made from materials readily obtained. The indicators can also now be purchased in this country.

SECTION VII. NOMENCLATURE OF INDICATORS

It is desirable, especially in purchasing indicators, that their chemical names should be used, since these names define the composition and to some extent the structure. Nevertheless

some of the chemical names are so verbose that they must inevitably be abbreviated in laboratory parlance. It is therefore desirable to agree upon some abbreviations.

Ortho-carboxy-benzene-azo-di-methyl-aniline is already known as methyl red. For the corresponding di-propyl compound we suggest "propyl red." Following a similar system we suggest for the sulfon-phthalein series of indicators the names which are listed in table 2.

(To be continued.)

SOIL FLORA STUDIES[1]

PART I

H. JOEL CONN

New York Agricultural Experiment Station, Geneva, New York

Received for publication, November 28, 1916

GENERAL INTRODUCTION

During the past twenty years or more, considerable attention has been given to the relation of bacteria to nitrogen-transformations in soil and in manure. This particular phase of soil microbiology has been so extensively studied because of the manifest importance of nitrogen in the nutrition of plants. There still remain, however, many other problems connected with the microörganisms in the soil that are of fundamental importance and which must be solved before a true appreciation of the significance of soil bacteria to agriculture is possible.

There are, for example, many unanswered questions relating to the distribution, function, and associative action of the microorganisms known to be abundant in soil. These questions require a method of attack almost the opposite from that used in studying the nitrogen-transformations. The latter problem started with a known function and was directed toward the unknown organisms to which this function belonged; the other problem starts with known organisms and is directed toward their unknown functions. The study of nitrogen-transformations has been centered around a few special physiological activities; while the other problem is concerned with the general characteristics of the entire soil flora. Therefore, investigations of the latter sort may be distinguished from those of the former by calling them soil flora studies.

In making a study of the soil flora, the first step necessary is

[1] Original abstracts of Technical Bulletins of the New York Agricultural Experiment Station.

classification. Investigations relating to the distribution or function of the microörganisms cannot proceed far without a thorough knowledge of the kinds of organisms in question. It is not yet possible to make a complete classification of the microscopic flora of soil; but it is possible to make a preliminary grouping of the organisms. Nothing more than this is attempted in the present work; but it is hoped that this preliminary grouping may be used by later investigators and developed by them into a more extensive classification.

The results that are now ready for publication are to be embodied in a series of five papers, entitled:

I. The general characteristics of the microscopic flora of soil.
II. Methods best adapted to the study of the soil flora.
III. Spore-forming bacteria in the soil.
IV. Non-spore-forming bacteria in the soil.
V. Actinomycetes in the soil.

The papers are to be published in full as technical bulletins of the New York Agricultural Experiment Station, which are to appear simultaneously with these abstracts in the Journal of Bacteriology.

I. GENERAL CHARACTERISTICS OF THE SOIL FLORA

Previous investigations

Soil flora studies in the past—and the same thing is true concerning flora studies of milk or water—have developed in two different directions, the first extensive, the second intensive. Extensive studies have resulted in learning a little about many kinds of soil organisms; intensive studies in learning much about a few small selected groups of organisms. Both lines of work must be harmonized in order to obtain a comprehensive knowledge of the soil flora.

The most important contributions to the extensive flora studies of soil are those of Chester (1900, 1903, 1904) and of Hiltner and Störmer (1903). The contribution of Hiltner and Störmer is especially important because they pointed out three

large groups of soil microörganisms which may be readily distinguished on gelatin plates and showed that the relative abundance of these three groups is nearly constant in normal soil. From their data it appeared that any external influence which disturbed the equilibrium of the soil flora would be indicated by a change in the relative abundance of these three groups. This conception of the soil microörganisms as being normally in a state of equilibrium has proved of considerable value in interpreting soil phenomena.

Meanwhile intensive work on special groups of soil bacteria has been accumulating more rapidly than extensive work on the general flora. Part of this work has been done on the group of spore-forming bacteria, and part of it on the Actinomycetes. As these two groups are to be taken up in two of the following papers, a more complete discussion of the literature will be given in them.

Thanks to the intensive work which has been done on these two groups, extensive work on the general soil flora can be undertaken today with a much greater chance of success than in earlier years. Flora studies have proved of value in dairy work, having resulted in showing the significance of the lactic acid types in milk and cheese (in milk the common lactic acid type, and in cheese the common type and also the Bulgarian type). Knowledge of equal importance in regard to soil may easily result from a better acquaintance with the soil flora.

Realizing this, the writer several years ago criticized the prevailing methods of soil bacteriological investigations, pointing out the importance of making a flora study, and concluded with the words: "in the future, if we wish to make any great advance in our knowledge, we must learn first to distinguish as rapidly as possible the different bacteria present in any given soil and then must study the significance of the forms we have learned to recognize" (Conn, 1909). Following up the line of work indicated in this statement, a preliminary grouping of the bacteria in a certain soil was published a few years ago (Conn, 1913). This study included all the forms obtained from gelatin plates incubated in the presence of air. About thirty-five types

of bacteria were mentioned that could be distinguished by means of certain cultural and physiological characteristics. It was expected that some of these types could later be broken up into two or more species, while others would prove to be separated by characteristics not sufficiently constant to furnish actual distinctions. This study has been continued for five years since the work already published was brought to an end; and now considerable information is at hand to show which of these types are distinct, which must be grouped together, and which require further subdivision. During the course of this study, several minor points have been investigated which have been published from time to time. The surprisingly high plate counts of frozen soil, for example, were noticed early in the work, and as data accumulated on this subject, they were published (Conn, 1910, 1912, 1914 a; 1914 b). Gradually, as the work progressed, evidence was obtained as to the comparative value of different culture media for soil bacteriological work (Conn, 1914 c). These minor publications all had to do with quantitative work alone, although the main line of investigation was primarily qualitative. The reason for this was the greater ease with which results could be obtained in quantitative work. It is only recently that the qualitative side of the work has been advanced sufficiently for publication. Two short articles have been published within the last year, one dealing with the spore-formers of soil (Conn, 1916 a; 1916 b), and the other with Actinomycetes (Conn, 1916 c; 1916 d). The work published in all these papers formed an integral part of the general soil flora study; so it is all summarized in the present series of publications.

Soils investigated

During the course of this investigation, samples have been taken from as great a variety of soils as could be obtained in the neighborhood of the Experiment Station. This included muck, clay loams, loams, sandy loams, and fine sand. They were mostly agricultural soils, but some of the muck samples were from woodland. The samples were taken from soils in various

stages of cultivation. Many of the samples were so selected as to furnish a comparison between sod and cultivated portions of the same soil.

Kinds of microörganisms in soil

Limitations of the study. At present it would be a hopeless task to make a complete study, or even a complete classification, of the various types of microörganisms occurring in soil. Because of the necessity of setting some limit to the work, only those forms have been studied that grow in the presence of air and occur in appreciable numbers on plates inoculated with soil infusion diluted 100,000 times. This excludes a number of the soil bacteria that have been described by others.

The organisms concerned with the transformations of nitrogen are not mentioned in this work because special media are necessary in order to obtain them. As all the plates were incubated in the presence of air, strict anaerobes, if such occur in soil, have been overlooked; but evidence has been obtained which indicates that they are not of much importance in soil. Two other classes of organisms that may be important in soil, which have been excluded from the present work, are higher fungi and protozoa; but it is still a matter of dispute whether these two groups are sufficiently active in soil to be of importance in comparison with bacteria.

A further limitation of the work has resulted from the fact that a complete classification of a flora as complex as that of soil—or even that part of it which develops on aerobic gelatin plates—offers difficulties that are almost insurmountable at the present day. The most that can be looked for at present is a preliminary classification. This preliminary classification should include descriptions of known species that can be recognized without question; but to describe new species seems unwise. The literature relating to soil bacteriology is full of names of species in regard to which too little information is given to make recognition possible. It seems unwise to increase this confusion by adding more new names. The best plan seems to

be to classify the soil microörganisms into groups or types as small as present methods allow, each of which may be the subject of future investigation. Some of these types are undoubtedly species; others are larger groups, and must be further subdivided in the future.

Peculiarities of the soil flora. One of the most striking characteristics of the soil flora is its uniformity. Quantitatively this uniformity is particularly striking, as the number of colonies developing on gelatin or agar plates, incubated for seven days, is ordinarily between five and fifty million per gram of soil. Qualitatively, there is also considerable uniformity. The characteristic microscopic flora of soil, is different from that of any other natural medium. The following groups of microörganisms almost always develop on aerobic gelatin plates inoculated with soil:

1. From 5 to 10 per cent spore-formers (The *B. subtilis* group). Nearly all the colonies of these bacteria, however, seem to come from spores instead of from active organisms.

2. Under 10 per cent rapidly liquefying, non-spore-forming, short rods with polar flagella (principally *Ps. fluorescens*).

3. From 40 to 75 per cent slowly liquefying or non-liquefying, non-spore-forming, short rods.

4. A few micrococci. In cultural characteristics these are almost identical with the last mentioned group.

5. From 12 to 50 per cent actinomycetes.

Of these five groups, the most important ones seem to be numbers 3 and 5. This conclusion is based upon the following facts: these two groups are always present in large numbers; of the other three groups, the only one always present in large enough numbers to be detected on the plates is number 1, the group of spore-formers, which apparently exists in normal soil only in the form of spores, and hence can not be active there.

The following groups seem to be lacking or to be present in such small numbers that they have been overlooked: organisms producing gas from sugars; non-liquefying bacteria producing acid from sugars; non-spore-formers that can grow in sugar broth in the absence of free oxygen.

II. METHODS BEST ADAPTED TO THE STUDY OF THE SOIL FLORA

Introduction

Methods for use in bacteriological investigations of soil are still in an experimental stage. Various methods have been suggested for different purposes, but none of them have been standardized. For the purpose of making a flora study of soil, it has proved necessary to develop many new methods.

The poured plate of agar or gelatin has been used as the basis of the present flora studies. Plate culture is useful for two purposes: it gives some idea as to the number of microörganisms in the soil; and it serves as a basis for qualitative work.

Plating soil for quantitative purposes

When using the plate method for quantitative work, the important points in technic are the temperature used for incubation and the length of time the plates are incubated. Fairly low temperatures (18°C. for gelatin and not over 25°C. for agar) and at least seven days incubation have been found to give the best results. The exact composition of the medium used (within certain limits) does not seem to have much influence upon the count obtained. This matter was taken up in a previous publication (Conn, 1914 c), in which it was stated that if anything the highest counts were obtained upon a soil-extract gelatin; but an asparaginate agar was especially recommended, principally because of its definite chemical composition. Since that paper was published further modifications of both media have been made.

A number of comparisons have been made between soil-extract gelatin and tap-water gelatin, showing that tap-water can be substituted for soil-extract without affecting the results obtained. Some work has also been done to determine what concentration of gelatin is best. Besides Gold Label Gelatin, which was used throughout the earlier work, two other commercial brands of gelatin have been studied: one prepared for bacteriological work by the United States Glue Company of Milwaukee, the other,

called "Bacto-Gelatin," prepared by the Digestive Ferments Company of Detroit. Both of these brands of gelatin have a higher jellying power than Gold Label Gelatin; so that if they are made up in a strength of 10 per cent about as firm a jelly is formed as with 15 per cent Gold Label Gelatin. The best quantitative results were obtained with 12 per cent United States Glue Company gelatin; but 12 per cent Bacto-Gelatin and 20 per cent Gold Label Gelatin gave high enough counts so that they can also be recommended.

The asparaginate agar has been modified by the addition of 10 grams of glycerin per liter. This was found to favor the Actinomycetes, to stimulate chromogenesis and to increase the size of the colonies to such an extent that it was immediately recognized as an improvement provided it gave a count as high as the unmodified formula. A series of soils was therefore plated on the two media, and the counts were as good or better with the glycerin formula.

As a result of these tests, tap-water gelatin and asparaginate-glycerin agar are recommended for quantitative work. The formula recommended for tap-water gelatin is:

Tap-water, 1000 cc.
Gelatin { 200 grams of Gold Label Gelatin
or 120 grams of Bacto-Gelatin or of United States Glue Company Gelatin.

Reaction adjusted to 0.5 per cent normal acid to phenolphthalein. (This will require about 20 to 30 cc. normal NaOH for Gold Label Gelatin, but under 10 cc. with either of the other brands.)

Clarified with white of egg in case Gold Label Gelatin or United States Glue Company Gelatin is used, but unclarified if Bacto-Gelatin is used.

The formula recommended for asparaginate-glycerin agar is:

Distilled water 1000 cc., agar 12 grams, glucose 1 gram, sodium asparaginate 1 gram, glycerin 10 grams, $NH_4H_2PO_4$ 1.5 grams, $CaCl_2$ 0.1 gram., $MgSO_4$ 0.2 gram., KCl 0.1 gram, $FeCl_3$ trace. Reaction adjusted to 1.0 per cent normal acid to phenolphthalein.

Whether the tap-water gelatin or the asparaginate-glycerin agar should be used will depend upon the conditions under which the work is being done. The counts obtained upon the

two media are nearly enough alike so that if a medium for quantitative work alone is desired, the choice between them should rest upon other considerations such as whether low temperatures are available for incubation.

Plating soil for qualitative purposes

When using the plate method as the basis of qualitative work, the composition of the medium is of much greater significance. No single medium has been found upon which all kinds of soil microörganisms can be recognized by means of their colonies. The best procedure at present available for making a qualitative study requires the use of both tap-water gelatin and asparaginate-glycerin agar. In the gelatin medium it is advisable to use 15 to 18 per cent of the Gold Label brand instead of 20 per cent as for quantitative work. On this gelatin the number of colonies of *B. megatherium*, *B. cereus*, *B. mycoides* and *Ps. fluorescens* can be counted. Gelatin also shows the relative numbers of Actinomycetes and lower bacteria, as even the most minute colonies of the Actinomycetes can be distinguished on gelatin (with a low-power microscope) from the colonies of lower bacteria. On asparaginate-glycerin agar it is possible to count the colonies of several types of Actinomycetes, although unfortunately this medium fails to show any difference between two of the most common types. The different kinds of non-spore-formers can not be recognized upon any of the media yet investigated—with the exception of *Ps. fluorescens*, which is not one of the most abundant types.

A long incubation at comparatively low temperatures is so very important in qualitative work that it needs special emphasis. Colonies of the more slowly growing organisms do not begin to appear until the fourth day; counts made on the fourth day or earlier include only a very small proportion of the Actinomycetes and almost none of the large group of slow-growing non-spore-formers. A qualitative study of soil bacteria based on plates incubated for such a short time would give a very erroneous conception of the actual soil flora.

For a complete qualitative analysis of the soil flora, at least three media may prove necessary in plating, one of them adapted to bring out the distinctive characteristics of the common soil types of each of the three groups of soil microörganisms. By plating soil on three such media, it would then be possible to recognize at a glance the most abundant types. The types that can not be recognized by means of their colonies should be isolated and studied in pure culture.

The study of pure cultures

Pure culture studies of about a thousand cultures isolated from soil have been made in the course of the present work. Various methods have been used in these studies. The classification card of the Society of American Bacteriologists has proved quite well adapted to the study of the spore-formers; and has proved of value in making a preliminary study of other organisms before learning what special tests were best adapted to them.

Certain of the tests called for in studying an organism by means of the classification card have appeared to give sufficiently consistent results to be of diagnostic value. These are: shape of vegetative forms; size of vegetative forms; arrangement of flagella when present; presence of spores; size and shape of spores; growth in the absence of oxygen; liquefaction of gelatin; nitrate reduction; chromogenesis; and form of growth in solid or liquid media. Even in regard to these points, however, inconsistent results are often obtained, a fact which must be taken into consideration when using the data as a basis for classification.

Other tests called for on the classification card have proved to give such inconsistent results as to be of little diagnostic value. These are: presence or absence of flagella; fermentation of sugars; diastatic action on potato starch; fermentation of glycerin; production of indol; and action on milk. In some cases it is possible that the inconsistent results of these tests are due to actual physiological variations in the bacteria; but it is felt that more often they are caused by imperfections in the technic at present available for making the tests.

The standard tests have proved of least value for the study of the Actinomycetes and slow-growing non-spore-formers. These two groups grow very poorly in ordinary media and nearly always give negative results in the tests called for on the classification card. In studying the Actinomycetes the most important information was obtained from a study of their growth on various special agar media containing little or no protein material. No methods have yet been found which prove satisfactory for the qualitative study of the slow-growing non-spore-formers.

(To be continued.)

REFERENCES

CHESTER, F. D. 1900, 1903, 1904 Del. Agr. Exp. Sta. Rept. 11, 52–75; Rept. 14, 52–66; Bul. 65.

CONN, H. J. 1909 Future methods of soil bacteriological investigations. Centbl. Bakt., II Abt. **25,** 454–457.

CONN, H. J. 1910, 1912, 1914 a, 1914 b Bacteria of frozen soil. Centbl. Bakt., II Abt., **28,** 422–434; **32,** 70–97; **42,** 510–519. N. Y. Agr. Exp. Sta. Tech. Bul. 35.

CONN, H. J. 1913 A classification of the bacteria in two soil plats of unequal productivity. Cornell Agr. Exp. Sta. Bul. 338, 65–115.

CONN, H. J. 1914 c Culture media for use in the plate method of counting soil bacteria. N. Y. Agr. Exp. Sta. Tech. Bul. 38.

CONN, H. J. 1916 a, 1916 b Are spore-forming bacteria of any significance in soil under normal conditions? N. Y. Agr. Exp. Sta. Tech. Bul. 51. Jour. Bact., **1,** 187–195.

CONN, H. J. 1916 c, 1916 d A possible function of Actinomycetes in soil. N. Y. Agr. Exp. Sta., Tech. Bul. 52. Jour. Bact., **1,** 197–207.

HILTNER, L. and STÖRMER, K. 1903 Kaiserliches Gesundheitsamt, Biol. Abt. Land- u. Forstw. **3,** 445–545.

THE INFLUENCE OF MILK AND CARBOHYDRATE FEEDING ON THE CHARACTER OF THE INTESTINAL FLORA

IV. DIET VERSUS BACTERIAL IMPLANTATION

THOMAS G. HULL AND LEO RETTGER

From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University

Received for publication, June 16, 1916

This paper marks the culmination of several years of investigation carried on in this laboratory on the relation of diet to the intestinal flora. The results given in earlier publications (Hull and Rettger (1914); Rettger and Horton (1914); Rettger, (1915) may be summarized briefly as follows.

1. A change in the diet of white rats from the ordinary mixed food to a special diet containing starch, lard, protein-free milk and a pure protein quickly resulted in a marked simplification of the intestinal flora, the Gram-positive organisms increasing, often to the extent of constituting the entire flora. *B. coli* and other intestinal organisms were practically eliminated, while bacilli of the type of *B. acidophilus* (Moro) predominated or were present in such numbers as to exclude all other forms.

2. *B. acidophilus* was found to be a common inhabitant of the intestinal tract of white rats under certain conditions of diet. It was increased in numbers by grain feed (particularly oats, wheat and corn), milk, and lactose. Its preponderance over other organisms was brought about within a period of two to four days when lactose was fed. It was often supplanted by *B. bifidus* which on continued lactose feeding persisted. In milk feeding the acidophilus phase was more permanent, and *B. bifidus* seldom gained the ascendency. Carbohydrates, other than milk sugar, failed to bring about this transformation.

The ingestion of foreign bacteria, even in large numbers, did not of itself bring about an elimination or displacement of the common intestinal microorganisms. This was especially true of *B. bulgaricus*. Vastly more important is the influence of diet, particularly milk and lactose. The feeding of *B. bulgaricus* tablets without the use of milk or lactose can therefore be of little, if indeed of any, value.

The work upon which this paper is based was largely a continuation of the earlier experiments on milk and carbohydrate feeding in which white rats were used almost exclusively. Particular emphasis was placed, as heretofore, on organisms of the aciduric group of bacteria, especially *B. acidophilus* and *B. bifidus*. Numerous comparative tests were made with these two organisms and *B. bulgaricus*. Feeding experiments were conducted on man as well as rats. More extended studies were made of the influence of a starch diet on the intestinal bacteria, and on the amylolytic or starch decomposing bacteria, particularly *Glycobacter peptolyticus* of Metchnikoff. Furthermore, tests were made for the presence of lactose and increased acidity in the digestive tract, after carbohydrate feeding.

In the present investigation 180 individual experiments were conducted on white rats, four on man, and six on other laboratory animals. In many instances the same animals were used more than once.

A more satisfactory method of collecting the feces of white rats was employed than has been described in previous papers. The rats were held by the tail and gently rubbed on the back just above the base of the tail. In this way the samples could be collected into test tubes containing sterile water or physiological salt solution. The methods of feeding, and the technique in general were essentially the same as those used heretofore.

For the detection of starch-attacking organisms, starch-agar plates were used, as originally suggested by Eijkmann (1901). This medium at first contained 0.1 per cent each of peptone and meat extract, but later both were omitted, the only ingredients being 1 per cent each of corn starch and agar. The starch

digesters on the plates showed a clear zone around the colony which failed to turn blue on the application of iodine solution (Lugol). Tests for the Welch bacillus were made by inoculating whole milk and heating the tubes at 80°C. for fifteen minutes. Sufficient anaerobiosis was provided for by the cream layer. *B. coli* was detected by its colonies on the agar plates and by gas formation in Veillon tubes. Lactose bile tubes were also used at times, as well as the litmus-lactose-asparagin-agar medium recommended by Ayers (1915).

The diet of the rats, unless otherwise noted, consisted of bread and green or vegetable food. Milk was supplied in small dishes, and the lactose and other carbohydrates were usually given in dry form on the bread or bread crumbs. The bacteria which were fed were either suspensions of twenty-four slant glucose agar growths in water or, as in the study of the glycobacteria, plain bouillon cultures or the washings of plain slant agar growths.

MILK FEEDING

In the later experiments conducted on white rats results were obtained which were in full agreement with those already published. In addition to the ground covered heretofore the influence of milk feeding on the intestinal flora of rats which were supplied with a high meat diet was determined. The results of these experiments may be stated as follows:

1. Sweet milk when added to a diet of bread and green food or vegetables favors the multiplication of the aciduric group of bacteria in the intestine of the white rat. During long continued milk feeding *B. bifidus* of Tissier may be present in large numbers, but as a rule *B. acidophilus* of Moro is the predominating organism.

2. While a marked transformation of the flora takes place complete simplification is rarely brought about by the use of milk alone.

3. The addition of milk to an established high meat diet does not lead to the multiplication of the aciduric group of bacteria to such an extent that the so-called "putrefactive" bacteria (*B. coli* and *B. welchii*) are eliminated.

CARBOHYDRATE FEEDING

Some of these experiments were merely a repetition of the earlier work on carbohydrate feeding, and were entirely corroborative. In all of the work on carbohydrate ingestion no other sugar besides lactose was found to exert any apparent influence on the intestinal bacteria, with the exception of dextrine, and in only two instances was any change brought about by dextrine. In these rats, C20 and C21, the flora was by no means simplified, nor did *B. acidophilus* appear prominent. In the smears there were seen, however, short thick rods with pointed ends which were Gram-negative, and which were in marked contrast to the flora at all other times. The remaining six rats which received dextrine did not show this change.

In all of the work on lactose feeding only one rat failed to respond in the usual way. During a period of thirty days while the lactose was supplied neither *B. acidophilus* nor *B. bifidus* could be found in the smears, plates or Veillon tubes made from this animal. The results with all of the other rats which were fed lactose were quite consistent, as in previous investigations. *B. acidophilus* soon made its appearance, and unless supplanted by *B. bifidus*, during prolonged lactose ingestion, it remained the predominant, and as a rule practically the only organism occurring in the feces. In a number of instances, however, *B. bifidus* assumed the chief rôle of importance.

LACTOSE VERSUS A HIGH MEAT DIET

White rats which had been on a meat diet (10 to 15 grams of chopped beef daily) were given the customary amounts of lactose, 4 to 5 grams per day. These rats at the beginning of the lactose feeding harbored a typical meat diet flora, *B. coli* and *B. welchii* being prominent, and *B. acidophilus* apparently absent.

In rat B7 which had been on the meat diet for nine days before the lactose was fed a gradual simplification of the intestinal flora took place, and by the twelfth day practically no organisms were present but *B. acidophilus*. The meat and lactose feeding were continued for one month, at the end of which time

the rat was killed. *B. acidophilus and B. bifidus* were found to be present throughout the length of the intestine, but, in the colon a few other forms were observed.

Rat B9 having been on the meat diet for two months was given lactose for eight days. There was a change from the typical mixed meat flora to one in which *B. acidophilus* predominated, although a few other forms, particularly *B. coli*, were still present. On post-mortem examination *B. acidophilus* was found almost pure in the upper small intestine, but mixed with other forms of bacteria in the ileum and colon.

Rat B19 was fed nothing but meat for ten days, after which it received a meat-lactose diet for eighteen days. Within five days *B. welchii* was eliminated and the other organisms reduced in numbers, even *B. coli* being detected with difficulty. The feces contained *B. acidophilus* in almost pure culture. Rat C23 having received nothing but meat for nine days, was placed on a meat-lactose diet for thirty-five days. The fecal flora changed from a strictly meat to the lactose type, but at no time were gas formers absent from the Veillon tube.

Rats C4, C7 and C10 were given meat for eleven days, the intestinal flora changing to the high meat diet type. On the addition of lactose to the food there was a marked change toward the aciduric type of bacteria, although *B. coli* was still present at the end of a week. Rats C16 and C17 were kept on a meat diet for two weeks, at the end of which time a typical meat flora was established. On changing the diet to one of lactose and bread a change to the lactose type of fecal bacteria occurred.

In a number of instances lactose was first supplied to the rats, and as soon as an aciduric flora was established meat was added to the diet in the amounts stated before, in place of the bread. Rats C5 and C6 having gone on a lactose-bread diet for twelve days, received meat in place of the bread. During the two weeks that the meat was fed little change took place in the fecal flora, although a small number of colon bacilli were detected from time to time. On discontinuing the lactose feeding there was a rapid change in the flora to the mixed type. In

rat C8 which had been fed a lactose-bread diet for five days a non-aciduric flora was established after there was a change to a strictly meat diet.

MINIMUM LACTOSE REQUIREMENTS

The amounts of lactose required to bring about complete transformation of the flora varied with the different animals. As a rule the amount of lactose contained in the milk which was consumed was not sufficient. Few of the animals took more than 20 cc. a day, and some took less than half of that quantity. The total amount of milk sugar consumed was from about 0.5 to 1 gram daily.

Five rats which had been on a high lactose diet for some time were given 1 gram of the sugar daily with 12 grams of bread crumbs. After twelve days one rat had a mixed flora, although acidophilus bacilli were still numerous. In the remaining five animals there was a less pronounced change, *B. acidophilus* and *B. bifidus* still being the predominant types. After increasing the daily amount of lactose 1 to 2 grams per week there was an almost complete reversion to the pure, simplified type.

Two rats which had been on the regular stock diet were each given 2 grams of lactose with 10 to 12 grams of bread crumbs daily. The typical mixed diet flora was transformed in a few days to one of an almost purely aciduric character. It appears, therefore, as if 1 to 2 grams of lactose per day are sufficient to bring about simplification, although complete transformation requires amounts somewhat in excess of 2 grams.

THE FATE OF LACTOSE IN THE INTESTINE OF THE WHITE RAT

A number of rats which had been on a high lactose diet (dry) for at least three or four days were killed and thorough examinations of the intestine made for the presence of acids and unchanged lactose. For this purpose samples of intestinal contents from different portions of the intestine were weighed, emulsified in distilled water and tested as to acidity and milk sugar. For determining the acidity the suspensions were ti-

trated against phenolphthalein with twentieth normal sodium hydroxide. No unusual acidity of the intestine could be noted (see page 55).

In the first tests for sugar the emulsions were filtered and the Fehling test applied to the filtrate. The results were negative. In the next experiments the samples of well shaken material from the intestine were boiled vigorously before filtering, and relatively large amounts of the filtrate employed for the Fehling test. The results were, with very few exceptions, positive. According to these results the lactose must have been present in the intestine in a solid or undissolved form, and required heating to bring it into solution so that it would appear in the filtrate in sufficient amount to give an unmistakable reaction with the Fehling solution.

Sugar was found in different parts of the intestine of rats to which it was fed in dry form; also at times in the voided feces. Hence, the conclusion may be drawn that in the feeding of a high lactose diet some of the lactose is not absorbed from the intestine, and that the unabsorbed remnant serves as favorable food for the aciduric type of bacteria. This view seems justified, at least in so far as the feeding of lactose in the dry form is concerned.

Several experiments were conducted on rats which received the lactose in concentrated aqueous solution (one part of lactose to seven parts of water) in daily amounts containing 4 to 5 grams of the sugar. When a typical simplified flora was established and within three to four hours after lactose feeding the animals were killed. Without exception, lactose was found in the intestine. Unboiled filtrates from the stomach and jejunum contents of one rat gave positive Fehling tests, while those from the duodenum, ileum and colon were negative. Boiling of the suspension from the last three sources made no difference in the findings. Similar results were obtained in a second rat, with the exception that the lactose was found as far down as the ileocecal valve. In two other rats sugar was detected in solution in the stomach, jejunum and ileum, but not in the duodenum and colon.

According to these results lactose, when fed in solution, is absorbed from the digestive tract before it reaches the lower intestine. Therefore, if lactose is acted upon by the aciduric group of bacteria, the decomposition must take place above the colon, unless such small amounts of lactose reach the colon as to escape detection by the method employed.

The following conclusions may be drawn regarding lactose feeding:

1. The ingestion of lactose in sufficient amounts will completely simplify the intestinal flora of the white rat, reducing it to the purely aciduric type.

2. The simple character of the flora remains permanent during continued lactose feeding.

3. The results are the same whether lactose is fed in a dry state or in solution. When dry sugar is employed it may be detected throughout the length of the intestine and in the feces, by means of the Fehling test. When fed in solution, however, its presence could be demonstrated only as far as the ileocecal valve.

4. Whether the regular diet is of the usual mixed type or very rich in protein, as for instance meat, lactose, when fed in large enough amounts, will exert the same simplifying action on the bacteria of the intestine. The putrefactive influence ordinarily brought about by a rich meat diet is, in the white rat, entirely neutralized by the lactose feeding.

5. The daily amount of milk sugar required to bring about a complete simplification of the flora varies somewhat with the individual rats. The average quantity, however, when a mixed diet is supplied is between 2 and 3 grams.

THE INFLUENCE OF LACTOSE FEEDING ON THE CHEMICAL REACTIONS OF DIFFERENT PARTS OF THE DIGESTIVE TRACT

In the experience of the writers all strains of acid-tolerant intestinal organisms are low acid producers, at least as compared with a typical strain of *B. bulgaricus*, and may be classed with the group of lactic acid bacteria designated the "Paratype"

or Type "B" of White and Avery (1910). *B. bifidus* has seldom produced an acidity in excess of 0.7 per cent, while the maximum for *B. acidophilus* has on some occasions reached 1.5 per cent. *B. bulgaricus* Massol, or Type "A," frequently attains a maximum acidity in milk of at least 3 per cent.

Acidity tests were made on the intestinal contents of 29 rats, 17 of which had been on the regular diet and possessed the usual mixed flora, while the remaining 12 had been fed lactose and harbored a simplified flora. Different parts of the digestive tract were tested by the method already described on page 53, in connection with the Fehling reaction for sugar.

While there were differences in the acidity of the individual rats, the acidity curve of the lactose animals runs practically parallel with that of the rats having the usual mixed diet. However, the average acidity of the 12 lactose-rats was less than that of the other group, which is contrary to what might be expected. It is safe to conclude from these experiments, that the acidity in the digestive tracts of the rat is not increased by lactose feeding under the conditions of these experiments.

INFLUENCE OF LACTOSE FEEDING ON MAN

Since the digestive processes of mammals are more or less alike, the ingestion of milk sugar in man might be expected to have the same effect upon the intestinal bacteria as in the rat. This was found, in a measure at least, to be true.

Four human adults consumed milk sugar with the daily diet for varying lengths of time. The diet varied, but in every instance the protein intake was reduced below the average, and usually considerable milk was added.

Subjects H1 and H2 were on the lactose regimen for eleven days. The average meal consisted of two slices of bread with butter, one quart of milk and all the lactose that the subjects cared to consume. Very little fruit was eaten. During the first two days four moderate doses of Epsom salts were taken by H1, but without any resultant diarrhea. During the eleven days this subject consumed 8 pounds of milk sugar. At the begin-

ning of the experiment the intestinal flora was typical of a healthy individual on a mixed diet, no acidophilus bacilli being demonstrable. On the third day the flora began to change, as evidenced by microscopical examination of slides, large Gram-positive rods becoming more numerous. On the seventh day the change was quite marked, acidophilus bacilli making their appearance in the agar plates. By the tenth day *B. acidophilus* was quite plentiful in the plates, with few other organisms accompanying it. *B. bifidus* was present in large numbers in the Veillon tubes, while on the slides large Gram-positive rods made up almost the entire microscopic picture.

Subject H2 consumed only 4 pounds of sugar in the eleven-day period, and took no laxative. On the third day after the beginning of the experiment a few acidophilus-like rods appeared in the smears, but their increase in number was very slow. By the eleventh day, however, the flora became somewhat simplified, with *B. acidophilus* occurring in moderate numbers, but no indication of *B. bifidus* colonies in the Veillon tubes.

The diet of the other two subjects could not be so easily controlled. H3 consumed a small amount of lactose and milk for two weeks, with his regular diet. There was but little change in the flora. For the next two weeks large amounts of lactose and milk were taken, other food being reduced in proportion. A marked change took place in the character of the feces and in the flora. The feces became light yellow in color, with a butyric consistency, and *B. acidophilus* became quite apparent in the smears and on the plates.

H4 on a mixed low protein diet, consumed four pounds of lactose during a period of two weeks. By the end of the first week *B. acidophilus* appeared both in the smears and on the plates. During this week a laxative had been taken causing some diarrhea.

In the following experiment the influence of a laxative upon the intestinal flora is brought out still better. Subject H1 on an ordinary mixed diet with low protein intake, consumed three pounds of lactose a week for a period of one month. Aciduric organisms (acidophilus and bifidus) were easily demonstrated

microscopically and culturally. Following the use of laxatives these organisms were present in much larger numbers. Without the ingestion of lactose no such changes were observed.

It seems probable that the laxative hurried the lactose along so that a sufficient amount of it reached the lower parts of the intestine where the aciduric bacteria multiply most abundantly under the right conditions of nutriment and environment. Without the use of a laxative or some other agent which saves the lactose from rapid absorption in the small intestine but little of the sugar reaches that part of the intestine in which the most marked bacterial changes, putrefactive and otherwise, take place and hence little encouragement is given to the aciduric type of bacteria.

The above results are somewhat analogous to those of Tonney, Caldwell and Griffen (1916) who, in the examination of typhoid carriers found that the typhoid bacillus is more easily recovered from the feces after the use of a cathartic. They appear to be strongly of the opinion that typhoid bacilli are destroyed in the intestine unless hurried along by increased peristalsis.

INFLUENCE OF STARCH FEEDING ON THE INTESTINAL FLORA

In the earlier experiments no perceptible change was observed in the character of the intestinal flora after the ingestion of moderate amounts of starch (1 to 2 grams daily). Since then similar experiments have been conducted in which larger quantities (4 to 6 grams) of starch were fed and in which starch-agar, in place of the regular agar or glucose-agar, plates were employed for the detection of amylolytic bacteria.

Rats C41 and C42, having previously been on the stock diet of bread and vegetables, were supplied with liberal amounts of corn starch together with ground bread. At the beginning no starch-attacking colonies were obtained from the feces. Within four days the plates showed numerous colonies of amylolytic organisms with their characteristic clear zones which on iodine treatment remained colorless. Rat C17 was given a mixture of corn starch and ground bread, equal parts by weight, for fif-

teen days. No starch decomposing bacteria could be demonstrated in the feces at the beginning. Within three days, however, numerous colonies of this type made their appearance on the plates. These organisms remained conspicuous throughout the experiment. In three to four hours after the last starch feeding the rat was killed. Starch was found in the stomach, but not beyond. On microscopical examination of material from the intestine the ordinary mixed flora of stock rats was observed.

Rats C5 and C6 were supplied with corn starch and bread for 18 days, and gave practically the same results. Rat C37 received the same diet for 25 days. Amylolytic bacteria soon became apparent and remained prominent throughout the period, as shown by the starch-agar plates.

Control rats which were on the regular stock diet at no time harbored this type of bacteria in sufficient numbers to be detected by the plate or any other method. Nor was any such tendency shown during corn and wheat feeding. However, one of two rats given wheat flour yielded a considerable number of typical starch attacking colonies.

The above positive results were obtained with the starch-agar plates only. By the use of ordinary agar or glucose-agar plates little difference could be seen between the flora of stock and of starch-fed animals. Direct microscopic examinations also revealed little if any difference. The conclusion must be drawn, therefore, that the ingestion of starch, while it brings about a marked increase in the strongly amylolytic type of intestinal bacteria, does not materially alter the relative numbers and distribution of the common intestinal organisms. However, the results are such as to show that the feeding of starch has a more marked influence upon the flora than the mere feeding of large numbers of representative starch decomposing bacteria, of which *Glycobacter peptolyticus*, which has been employed by us, is a notable example.

GRAIN FEEDING

In these experiments 35 white rats were employed; wheat was given to 5, oats to 8, corn to 9, mixed grain feed to 5, and wheat flour to 5. The individual experiments lasted from a few days to three and a half months. A small number of tests were also conducted on the common domestic fowl. Without going into the details of this particular investigation the conclusions may be stated as follows:

The ingestion of wheat and of oats seems to favor in a small degree the multiplication of *B. acidophilus* in the intestine of the white rat. In no instance was *B. acidophilus* predominant, nor did the flora of the rats become appreciably less complex.

B. acidophilus is more or less abundant in the intestine of the common domestic fowl, and the temporary substitution of one grain in the mixed feed for another has but little influence upon the intestinal flora. Addition of milk, however, tends materially to increase the number of the aciduric bacteria.

Corn exerts little if any influence on the character of the intestinal flora. Wheat flour is likewise of little significance.

Mixed grains (wheat and oats, or wheat, oats and corn) affect the intestinal organisms in about the same manner and degree as oats or wheat, when these are fed alone.

INGESTION OF FOREIGN BACTERIA AND INTESTINAL IMPLANTATION

Repeated attempts to establish *B. bulgaricus* in the intestine by the ingestion of large numbers of these organisms were unsuccessful. Some of the decisive results obtained in earlier experiments have already been published, and little need be added here except that these results have been consistent throughout the investigation, and admit of but one conclusion, namely that the mere feeding of *B. bulgaricus* does not lead to the acclimatization of the organism in the intestine, even for a short time. The ingestion of milk, raw or sterile, brought about a change in the aciduric type of flora, but this type was represented by *B. acidophilus* of Moro, and not by the bacillus of Massol, as was readily shown by certain cultural tests, par-

ticularly the character of the agar colonies, and by the slowness with which litmus milk was acidified.

Similar experiments were conducted with *Glycobacter peptolyticus*, an active starch-digesting organism which was originally isolated by Metchnikoff from the intestine of a dog, and which has been advocated by Metchnikoff and others as an important aid in bringing about the acclimatization of *B. bulgaricus* in the intestine. It is claimed that this organism attacks the starches and thus makes them available for utilization by *B. bulgaricus* (Wolman, 1912).

Several experiments involving the use of *Glycobacter peptolyticus* were carried on with white rats. As this organism readily produces spores difficulty was at first encountered in the interpretation of results. It was but natural to expect spores to be recovered from the intestine, even though very young cultures were fed.

SPOROGENOUS CULTURES OF GLYCOBACTER PEPTOLYTICUS

As a rule abundant spore formation took place in cultures of *Glycobacter peptolyticus* within twenty-four to thirty-six hours when the organism was grown at 37°C. on plain agar. Water suspensions of the bacilli and spores were prepared from forty-eight hour slant agar tubes, and poured over the bread which constituted the chief diet of the rats. The feces were examined at regular intervals. In order to distinguish between vegetative and spore forms of the glycobacter two sets of tests were made; in one of these the fecal suspensions were heated at 85°C. for fifteen minutes, while in the other the heating was omitted.

In the first experiment two white rats were employed. During the twelve days that the experiment continued there was a small number of amylolytic organisms on the starch-agar plates. The colonies from the unheated suspensions were but slightly in excess of those obtained after preliminary heating.

In the second experiment four rats were fed glycobacter suspensions for five days. A small number of starch-decomposing organisms was obtained from the feces. Heated and unheated

suspensions showed some differences, however. In most instances the counts on the plates of the latter were much lower than on the other set, the reduction amounting to as much as 17 to 1, 24 to 3, and 20 to 2. Vegetative forms were undoubtedly present in the feces.

In the third set of experiments eight rats received spore suspensions for eleven days. At the end of this period glycobacter was present in the feces in comparatively large numbers, and mostly in the vegetative form. It did not persist long, however, as in three days after the spore feeding was discontinued very few colonies of starch attacking bacteria could be obtained from three of the rats, while in the feces of the fourth no organisms of this type were demonstrable.

Since very few or no bacteria which are able to decompose starch were present in the feces of the above animals before the beginning of spore feeding, it must be concluded that development of at least some of the spores must have taken place in the intestine, presumably the large intestine. Whether any multiplication of the organism occurred in the intestine is wholly a matter of conjecture. It may be assumed, however, that the organism is able in the spore form to pass through the stomach and small intestine without serious injury, especially when the diet consists essentially of bread, which contains considerable starch. The above experiments are incomplete, and are to be resumed.

FEEDING OF ASPOROGENOUS SUSPENSIONS OF GLYCOBACTER PEPTOLYTICUS

Non-sporing growths of this organism were easily obtained by cultivating it on glucose agar. Suspensions of twenty-four hour asporogenous cultures were fed with the regular diet of bread and vegetables.

Rat B3 received large numbers of the organism poured over bread crumbs. The starch-agar plates showed a marked increase in the number of starch-digesting colonies. Somewhat similar results were obtained with rat C16. On the other hand, rat

B17 which was on a continued high meat diet and which received the same amount of bacterial suspension showed no increase in the number of amylolytic organisms in the course of five days of continued feeding. The small number of colonies of starch decomposing bacteria which appeared on the plates before the ingestion of the bacterial suspensions remained constant or was slightly reduced during the investigation.

Rats C1 and C2 were fed Glycobacter with bread crumbs for six days. No amylolytic organisms could be demonstrated in the feces before the ingestion of the glycobacterium. Following this, however, starch-attacking bacteria appeared in varying numbers, though they were never numerous.

In the next experiment four rats were given suspensions of Glycobacter for five days. During this period two of the rats yielded a small number of colonies on the starch-agar plates, but for only one day. In the feces of the other two rats no organisms of this type were observed.

Finally, twelve rats were employed in a series of tests. They were given asporogenous cultures, as before, daily for twelve days. During this entire period *Glycobacter peptolyticus* failed to establish itself in the intestine, as shown by the starch-agar plates, although on the eleventh and twelfth days a few starch-decomposing colonies were obtained. Two days later, however, no colonies appeared.

While the above results are not consistent they indicate that the mere feeding of *Glycobacter peptolyticus*, even in enormous numbers, does not bring about an established flora of starch-decomposing bacteria in the intestine of the white rat. In the later experiments the possible occurrence of spores was given greater consideration than before. All feedings were controlled by the previous microscopic examinations of the suspensions as to the presence of spores. Furthermore, it should be borne in mind that the diet in all but one of these experiments consisted almost entirely of bread which is rich in starch, and that on this diet, without the addition of bacterial cultures, few if any starch-attacking bacteria were present in the feces. The establishment of bacteria of this type should be made relatively

easier when large numbers of the organisms are ingested. In the single experiment in which meat was almost the entire diet no colonies whatever of amylolytic-bacteria were obtained from the feces either before or during the period of bacteria administration. These experiments are to be resumed, in order to obtain more conclusive results as to the fate of starch-decomposing bacteria under different conditions of diet, when introduced into the digestive tract.

Very definite results have been obtained with *B. coli*. This organism, although a normal inhabitant of the intestine of man and animals, does not increase appreciably in numbers in the intestine when ingested in large quantities. Rats A15 and A26, the intestinal flora of which had been simplified by continuous lactose feeding, were given, in addition to the lactose, twenty-four hour slant agar cultures of *B. coli* daily for a period of two and three weeks respectively. At no time were *B. coli* colonies obtained on the agar plates, nor was the simplified character of the flora of the intestine changed, in so far as microscopic examination could demonstrate. Even in a rat which had not received lactose for three weeks and which possessed the usual mixed flora, liberal feeding of *B. coli* for four days did not bring about a perceptible increase in the numbers of this organism. These findings were further corroborated by bacteriological examinations of the stomach and intestine of each of these animals, and are in full accord with those of Rettger and Horton (1914) and of Seiffert (1911).

Another well-known organism which is often found in the intestine, *Proteus vulgaris*, was employed by us in the same way. Two rats (C4 and C7) which had received no other diet besides bread and vegetable food, and the flora of which was of a typical mixed character, were fed large numbers of the organism in water suspension administered with the food, or by the pipette method. Very few of the organisms fed could be recovered from the feces by the usual method now adopted for this organism (gelatin tube culture and subsequent inoculation of slant agar condensation fluid). Even the substitution of meat for the bread in the diet did not make any material difference.

In his experiments on man and rabbits Kulka (1914) demonstrated that *B. metchnikovi* and *B. prodigiosus*, when introduced, either by mouth, or subcutaneous, intravenous or intraperitoneal injection, do not appear in the feces. Mitchell and Bloomer (1914) obtained similar results in the common domestic fowl with the typhoid bacillus.

Raubitscheck (1912) obtained positive results by first immunizing animals with the organism in question, by subcutaneous or intraperitoneal injection. When, after such preliminary treatment, the animals were given the organisms *per os* the bacteria were recovered in large numbers from the feces. He employed *B. prodigiosus*, *B. kiliense* and *M. cholerae*.

Similar experiments were carried on by the writers with white rats and guinea pigs. *B. acidophilus* and *B. bulgaricus* were used as the test bacteria. These were grown on slanted glucose agar and washed off with sterile water. Rat B3 was given four subcutaneous injections of *B. bulgaricus* suspensions in 1 cc. quantities at intervals of seven days. Rat B17 received four subcutaneous and three intraperitoneal injections of *B. acidophilus* in amounts varying from 0.5 to 1.5 cc. Rat B19 was given the same number of injections of another strain of *B. acidophilus* in amounts varying from 0.5 to 1.5 cc. Rat B19 was given the same number of injections of another strain of *B. acidophilus*, while a single guinea pig received the same treatment in which suspensions of *B. bulgaricus* were employed. Checks or controls were kept in the cages with each of these animals.

Within a few days after the last injection the different animals were fed suspensions of the same organisms with which they had been immunized. The results were uniformly negative, in so far as any appreciable change in the intestinal flora was concerned. However, since macroscopic and microscopic agglutination tests were negative, too much significance must not be attached to the results of this series of experiments. The organisms employed may not have been adapted to this line of investigation; or, the immunization of the animals may have been far from complete.

GENERAL DISCUSSION AND CONCLUSIONS

B. acidophilus (Moro) and *B. bifidus* (Tissier), two of the well-known members of the aciduric or lactic acid bacillus group of bacteria, are common inhabitants of the intestinal tract of the white rat and of man. At times their numbers may be so small that they escape detection without a most thorough search. Again, they may be very abundant and establish themselves for at least short periods of time to the exclusion of all other forms. The typical flora of infants which subsist on mothers' milk is a good illustration of the extent to which one intestinal organism (*B. bifidus*) may dominate and even supplant all other types. The most important factor in determining the character of the flora is the diet.

Lactose, milk and mixed grains (wheat, oats, etc.) are specific articles of diet which exert an influence on the intestinal bacteria. Lactose, when fed in sufficient quantities (two to three grams daily), brings about a complete transformation of the flora of white rats within two to three days: milk requires a longer time, and does not bring about a complete change. Milk and lactose together form the most practical and effective diet, at least for man. Grain feeds tend to increase the number of aciduric bacteria, but their influence is comparatively small.

Milk undoubtedly owes its beneficial action to the lactose which constitutes almost half of the solid matter present. The explanation of this action must lie in the fact that the lactose is absorbed slowly from the intestine. On several occasions it has been found in the feces of rats that had been supplied with it as a part of their diet. The raw grains are also probably acted upon slowly, or at least some of the intermediate carbohydrate products are not immediately absorbed. Bread, on the other hand, which contains cooked starch does not foster the development of the aciduric bacteria, because it is digested quickly, and no available sugar remains in the intestine long enough to be attacked and utilized by this group of bacteria.

The most satisfactory explanation of the favorable influence of lactose on the aciduric bacteria of the intestine must be

found in the fact that when lactose is present, even in minute quantities, optimum cultural and environmental conditions are created for these particular organisms, without a corresponding favorable change for the mass of other bacteria ordinarily present. That the action could not have been due to lactic acid produced from the sugar is strongly indicated by the absence of increased acidity in the intestine of the lactose rats, as compared with the controls. Rovighi (1892) showed that lactic acid, when ingested, had only a slight influence on intestinal putrefaction. Winternitz (1892) claimed that lactose when fed to animals or man exerted an inhibitory influence on putrefaction, but that this action was not due to lactic acid formed from the sugar, but to the lactose itself.

Meat or other high protein diet increases the indol-producing bacteria and other organisms of the so-called "putrefactive" type, like *B. coli* and *B. welchii;* cornstarch appears to foster the amylolytic group of intestinal organisms, while in a few experiments grain feed seemed to favor the development of what appeared to be fusiform bacilli.

The reaction of the intestine remained independent of the character of the intestinal flora. While the acidity of the intestinal contents varied in different rats, it was not increased during the course of lactose feeding experiments. The acidity was highest in the duodenum, as a rule, and lowest at the ileo-cecal valve.

It has been conclusively demonstrated in our work that a high lactose diet markedly influences the intestinal flora of man. These results have been fully confirmed recently by other investigators. Barker (1914) and Torrey (1915) report favorably on the use of lactose and milk in typhoid fever. Torrey demonstrated that the feeding of a high carbohydrate diet (milk and lactose) to typhoid patients tended to reduce the putrefying types of bacteria, and to encourage the acidophilic forms. Plain milk feeding was not as apt to do this as lactose. At least 250 to 300 grams of the milk sugar were required in most instances (daily) to produce a marked change in the intestinal flora of the patients, especially when the initial flora

was of a distinctively putrefactive type. In persons having non-putrefactive organisms predominant at the outset a radical change to an aciduric type was readily brought about by lactose ingestion.

The acclimatization of *B. bulgaricus* in the intestine after oral administration is a much-disputed point. The claims of Metchnikoff and his followers (Cohendy, 1906; Belonowsky, 1907, and Leva, 1908) that the ingestion of *B. bulgaricus* brings about an implantation of the organism in the intestine have not been substantiated by us. In our earlier work, as well as in the present investigations, we have been unable to establish this organism in the intestine of white rats even for short periods of time, although the bacilli were fed in large numbers.

Luerrsen and Kühn (1908) failed to implant *B. bulgaricus* in the intestine of man by the continued use of yoghurt. Similar results were obtained by Distaso and Schiller (1914) on white rats, although Distaso (1913–14) had observed in persons with cecal or ileum fistula that *B. bulgaricus* was present in the intestine in large numbers twenty-four hours after the use of milk which was soured with *B. bulgaricus*.

Rahe (1915) was unable to obtain evidence by feeding experiments that this organism can become adapted to the lower intestine of man. In monkeys *B. bulgaricus* appeared to be capable of a limited survival in the upper intestine. In a single experiment on a monkey Herter and Kendall (1908) found that by feeding the animal for two weeks on milk fermented with the bacillus of Massol an acid reaction was maintained throughout the intestinal tract, but that the bacillus in question failed to predominate. It was present, however, below the ileo-cecal region and in the duodenum and cecum, in almost pure form.

Attempts to establish *B. bulgaricus* in the intestine after immunization with the same organism were unsuccessful. Thus, claims made by Raubitscheck (1912) for other organisms have not been substantiated by us with the bacteria employed.

Glycobacter peptolyticus fails to establish itself in the intestine of the white rat, even when fed in large numbers. The bacilli may occur in varying amounts in the intestine when the food

consists largely of starch, as for instance bread; but on a rich protein diet very few are recovered from the feces. Spores of *Glycobacter peoptolyticus* may pass through the stomach and the small intestine unharmed, and may even undergo development into the vegetative form. Whether there is actual multiplication in any part of the digestive tract remains undetermined.

Attempts to acclimate foreign strains of *B. coli* and *Proteus vulgaris* in the intestine of the white rat failed completely. These results are in full accord with those of Seifert (1911) and others.

The results obtained by us on the influence of milk and lactose feeding are in at least partial agreement with the observations of the following investigators.

Weiss (1904) demonstrated the presence of large numbers of *B. acidophilus* in the intestines of persons not suffering with intestinal disturbances of any sort. Since most of these persons had consumed milk previous to the investigation Weiss assumed that the prominence of this organism bears some direct relation to milk diet. Lembke (1897) had shown that a marked difference may be brought about in the character of the intestinal flora by the substitution of a bread for a meat diet, though no prominence is given to organisms of the acidophilic type.

Herter and Kendall (1909) found that the intestinal flora of kittens and monkeys underwent a distinct change when the diet was changed from meat and eggs to milk and glucose. There was a substitution in the feces of an acidophilic type of bacteria for a flora that had been strongly proteolytic. They also observed a marked decrease in the intestine of indol, skatol, phenol and bound hydrogen sulphide, and of the indican and aromatic oxy-acids in the urine. In the light of our own experiments, it appears quite probable that the change in the nature of the flora was brought about by the milk, rather than by the glucose. Friedenwald and Leitz (1909) held that regulation of diet is the most logical way of controlling the intestinal bacteria. De Gaspari (1911) claimed that rats kept on a bread and grain diet showed a predominance of *B. bifidus* over *B. coli*, while meat favored *B. coli* and *B. welchii* to such an extent as almost to exclude *B. bifidus*.

The inhibitive influence of certain sugars and of milk on putrefaction has been known for many years. Hirschler (1886) appears to have been the first to demonstrate such action by carbohydrates. He showed that in vitro the common putrefactive products, indol, phenol, oxy-acids, etc., are held in check in a protein medium if cane sugar, lactose, dextrine, starch or glycerine is added. In the animal body the same results were obtained, but to a lesser degree, owing to the absorption of the carbohydrates from the intestine. The feces of dogs that were fed glycerine, sucrose and starch, together with 250 grams of meat, contained less indol than the control animals.

Rovighi (1892) found that a kephyr diet caused a great reduction in the ethereal sulphates of the urine, and of intestinal indol. He believed that acids played the important rôle of suppressing intestinal putrefaction, but could not substantiate this by practical experiment. Winternitz (1892) demonstrated that milk strongly inhibits putrefaction, and held that this was due to the lactose, and not to the acids resulting from its decomposition. The ethereal sulphates of the urine were reduced and there were other marked indications of lessened putrefaction.

Schmitz (1893) brought about a great reduction in the ethereal sulphates by feeding lactose. He believed, however, that the casein was the important factor in the milk and kephyr. The interesting observations of Bienstock (1901) and others that putrefaction of milk is prevented by lactose-decomposing bacteria are too well known to need comment; as are the repeated demonstrations that sugars are protein sparers in the presence of sugar-fermenting bacteria.

REFERENCES

Ayers, S. H. and Johnson, W. T. 1915 A bacteriological study of retail ice cream, U. S. Dept. Agric. Bulletin 303, 1–24.

Barker, L. F. 1914 The diet in typhoid fever. Jour. Am. Med. Assoc., **63**, 929–31.

Bienstock 1901 Milchfäulniss, Verhinderung der Fäulniss durch Milch. Arch. f. Hyg., **39**, 390–427.

Belonowsky, J. 1907 Influence du ferment lactique sur la flore des excrements des souris. Annal. de l'Inst. Pasteur, **21**, 991–1004.

COHENDY, M. 1906 De la desinfection intestinale obtenue, sans régime special, par l'acclimatation d'un ferment lactique dans le gros intestin. Compt. rend. Soc. Biol., **60**, 602–4.

DE GESPARI, F. 1911 Flore intestinale des rats blancs au régime ordinaire et au régime carne. Centralbl. f. Bakt. Abt. I Orig., **57**, 519–22.

DISTASO, A. 1913–14 Über das Schicksal der per os eingeführten Bakterien. Abstr. Centralbl. f. Bakt., Abt. I, Ref. **60**, 255.

DISTASO, A. ET SCHILLER, J. 1914 Sur l'acclimatation de microbes étrangers à la flore intestinale. Compt. rend. Soc. Biol., **76**, 243–4.

EIJKMANN, C. 1901 Concerning enzymes of bacteria and moulds, Centralbl. f. Bakt. **29**, 841–8.

FRIEDENWALD, J. AND LEITZ, T. F. 1909 Experiments relating to the bacterial content of the feces, with some researches on the value of certain intestinal antiseptics. Am. Jour. Med. Sci., **138**, 653–61.

HERTER, C. A. AND KENDELL, A. I. 1908 Fate of *B. bulgaricus* (in bacillac) in the digestive tract of a monkey. Jour. Biol. Chem., **5**, 293–302.

HERTER, C. A. AND KENDELL, A. I. 1909 The influence of dietary alterations on the types of intestinal flora. Jour. Biol. Chem., **7**, 203–36.

HIRSCHLER, A. 1886 Über den Einfluss der Kohlehydrate und einiger anderen Körper der Fettsäurereihe auf die Eiweissfäulniss. Zeitschr. f. physiol. Chem., **10**, 306–17.

HULL, T. G. AND RETTGER, L. F. 1914 The influence of milk and carbohydrate feeding on the intestinal flora of white rats. Centralbl. f. Bakt., Abt. I, Orig., **75**, 219–29.

KULKA, W. 1914 Studien zur Frage der fakalen Ausscheidung darmfremder Bakterien. Arch. f. Hyg., **82**, 337–50.

LEMBKE, W. 1897 Weiterer Beitrag zur Bakterienflora des Darms. Arch. f. Hyg., **29**, 304–53.

LEVA, J. 1908 Zur Beurtheilung der Wirkung des Lactobacillus und der Yogurtmilch. Berlin. klin. Wochenschr., **45**, 922–4.

LUERRSEN, A. UND KÜHN, M. 1908 Yoghurt, die bulgarische Sauermilch. Centralbl. f. Bakt., Abt. II, **20**, 234–48.

METCHNIKOFF, E. 1907 The Prolongation of Life.

MITCHELL, O. W. H. AND BLOOMER, G. 1914 An experimental study of the hen as a possible carrier of typhoid fever. Jour. Med. Res., **31**, 247–50.

RAHE, A. H. 1915 A study of the so-called implantation of *Bacillus bulgaricus*. Jour. Inf. Dis., **16**, 210–21.

RAUBITSCHECK, H. 1912 Zur Frage der Ausscheidung darmfremder Bakterien. Virchow's Arch., **209**, 209–20.

RETTGER, L. F. AND HORTON, G. D. 1914 A comparative study of the intestinal flora of white rats kept on experimental and ordinary mixed diet. Centralbl. f. Bakt., Abt. I, Orig., **73**, 362–72.

RETTGER, L. F. 1915 The influence of milk feeding on mortality and growth, and on the character of the intestinal flora. Jour. Exp. Med., **21**, 365–88.

ROVIGHI, A. 1892 Die Aetherschwefelsäuren im Harn und die Darmdesinfection. Zeitschr. f. physiol. Chem., **16**, 20–46.

SCHMITZ, C. 1893 Zur Kenntnis der Darmfäulniss. Zeitschr. f. physiol. Chem., **17,** 401–3.

SEIFERT, G. 1911 Fütterungsversuche mit körperfremden Kolistämmen. Deut. Med. Wochenschr., **37,** 1064–7.

TONNEY, F. O., CALDWELL, F. C. AND GRIFFIN, P. J. 1916 The examination of the urine and feces of suspect typhoid carriers with a report on elaterin catharsis. Jour. Inf. Dis., **18,** 239–46.

TORREY, J. C. 1915 The fecal flora of typhoid fever and its reaction to various diets. Jour. Inf. Dis., **16,** 72–108.

WEISS, H. 1904 Zur Kenntniss der Darmflora. Centralbl. f. Bakt., Abt. I, **36** Orig. 13–28.

WHITE, B. AND AVERY, O. T. 1910 Observations on certain lactic acid bacteria of the so-called bulgaricus type. Centralbl. f. Bakt. Abt. II, **25,** 161–78.

WINTERNITZ, H. 1892 Ueber das Verhalten der Milch und ihrer wichtigsten Bestandtheile bei der Fäulniss. Zeitschr. f. physiol. Chem., **16,** 460–87.

WOLMAN, E. 1912 The amylolytic bacteria of the intestine. Annal. de l'Inst. Pasteur, **26,** 610–24.

COCCIDIA IN SUBEPITHELIAL INFECTIONS OF THE INTESTINES OF BIRDS

PHILIP B. HADLEY

From the Agricultural Experiment Station of the Rhode Island State College[1]

Received for publication, July 10, 1916

In an article entitled "Aberrant intestinal protozoan parasitism" Dr. Theobald Smith (1916) has recently called attention to the presence of a subepithelial infection in a young turkey with a protozoan parasite the nature of which was not definitely ascertained. The presence of a contiguous infection of the intestinal epithelium with a species of coccidium suggested the possibility of the identity of the two forms, but Dr. Smith "is inclined to regard these epithelial cell parasites as belonging to a species distinct from that in the subepithelial tissues. . . . ." The question is raised, however, regarding the frequency of a subepithelial invasion among coccidia, and Dr. Smith suggests the possibility that such invasion might precede the earliest symptoms of the disease and might easily be overlooked.

For several years the present writer has been studying, when opportunity presented, the pathology of avian coccidiosis. The work is still in progress but since some time may elapse before publication it seems desirable to mention in a preliminary way the bearing of certain of the observations upon the problem brought forward by Dr. Smith.

In 1911, the writer (Hadley, 1911) described in detail the process of merozoite formation by the common coccidium of poultry, *Eimeria avium*. It was then stated that the "merozoite cysts" appeared to be of two kinds—one smaller and usually found in the epithelial cells of the upper intestine, the

[1]Contribution No. 222.

latter much larger, and occurring most frequently in the submucosa of the cecal walls. Dr. Smith's suggestion that such a phenomenon might represent in his case an "aberrant" type of infection has led the writer to a re-examination of material reported on in 1911, and to a closer study of other material from cases of coccidiosis in chicks and turkeys obtained since that time. The subject has assumed a special interest since Dr. Smith states that, in the case examined by him, the variation in the products of division, and the irregularity with respect to chromatin distribution leads him to assume that his subepithelial bodies (merozoite cysts?) were largely degenerating forms. This view, according to Dr. Smith "is supported by the abortive attempts at repeated multiplication within the primary cysts and by the partial disappearance of their contents."

The main point which the present writer wishes to bring out in this note is that, as he reported in 1909 and again in 1911, coccidia of the *Eimeria avium* type may be, and commonly are, found beneath the intestinal epithelium. This may occur sometimes in the duodenum, more frequently in the small intestine, and most commonly in the ceca. Coccidiosis in birds appears first in the majority of cases in the duodenum. If the bird survives this initial infection, the lower small intestine may be invaded; and finally the parasites gain lodgment in the ceca. The mature cysts, which are present in almost all poultry, young or old, if occurring in only small numbers in the cecal content or excrement, will usually be found to have had their origin in the epithelial cells of the duodenum. If they are present in large numbers (twenty to fifty per field with $\frac{1}{12}$ oil immersion and No. 4 ocular), the case is unusually severe and it then frequently happens that the epithelium of the lower small intestine and the ceca as well, have contributed to their formation. In the latter case sections of the cecum may show many crypts containing coccidia in various stages of development.

But with reference to the presence of coccidia in the subepithelial territory it is interesting to observe that they may be present there in large numbers even when the adjacent epithelial layers carry but a slight intracellular infection. The

writer has never, however, observed the presence of coccidia beneath the epithelium of the cecum without the presence of some of the same or other stages in adjacent epithelial cells. In many cases, however, the number of organisms located in the subepithelium is entirely out of proportion to the number in epithelial cells, either showing infection or indicating that they have been torn away from their base.

The stage of *Eimeria avium* most common below the epithelium is the "merozoite cyst," usually packed with ripe or immature merozoites. Less commonly the immature schizonts are observed. Macrogametocytes in which the wall has already begun to thicken are also found, but not often. Apparently the situation favors the development of the schizogonous cycle rather than the sporogonous. The merozoite cysts are frequently 50 to 60μ in length, and usually oval. They probably contain several hundred merozoites which are grouped about one or more bodies of reserve substance (Restkörper). Many of the cysts are certainly intracellular and all may be, although in sections it frequently happens that no limiting membrane or crescentic nucleus can be detected. The enclosing cells when present are undoubtedly endothelial in nature in many instances. In others the nature of the host cell has not been ascertained. Some are manifestly giant cells, the several nuclei of which can be observed pressed into a crescentic body at one side of the enclosed parasite. Even here, however, there is no indication that the parasites have been restrained in their development or have degenerated in any way. It seems probable that in some cases more than one merozoite cyst may occupy the same cell as is the case with epithelial cells in which four or five schizonts may frequently be seen.

The merozoite cysts of the submucosa are usually adjacent to the base of the crypts; less seldom in the core of the villi, although they may sometimes be packed in the core to the exclusion of nearly all other cell structures. In such cases they crowd closely on the basement membrane. They are often packed solidly along the muscular wall or in the muscularis mucosa, forming a definite barrier between the inner muscular layer and the

submucosa. In some cases the examination of transverse sections shows the parasites in this position through more than half of the circumference. At other times they are in isolated groups. They often lie closely packed together in a stroma or network of fibres from which most other cells have disappeared—probably from crowding out.

Judging from the number and size of these merozoite cysts, they are very far from being degeneration forms. The size of the merozoites, of which hundreds may be found in the spaces of the mucosa, is the same as that of the merozoites from the duodenum. They stain in the same way and give every evidence of complete development, whether they lie in the endothelial cells or lie separated from the enclosing cell. There is no indication of an "abortive attempt at multiplication."

Dr. Smith raises the question whether a subepithelial development of the coccidia may represent an early stage of infection. In most of the cases examined by the writer, studied by complete series of smears and sections from duodenum, intestine and ceca, the subepithelial infection cannot be regarded as "preliminary," in the sense that it precedes infection in other parts of the intestinal tract. In the cecum it is often present when the duodenum has already been ravaged by the attacks on the epithelium, and the ceca are beginning to show signs of infection in the epithelial cells. Sometimes much of the cecal epithelium in other regions has already suffered considerably and some of the villi are packed with cysts.

A point of considerable interest lies in the origin and manner of infection of the submucosa with the original merozoites that have formed the merozoite cysts. The fact that such infection is observed to exist behind apparently undamaged epithelial walls and is found more commonly in the submucosa adjacent to the bases of the crypts rather than in the cores of the villi, suggests that the parasites may become located here through the agency of the blood stream, having been taken into the circulation in the greatly damaged portions of the duodenum or perhaps in the cecum itself. Here capillaries are frequently broken into; seldom does a severe coccidial infection take place

without a greater or less haemorrhage into some portion of the intestinal canal.

On the other hand it is not impossible that the infection of the mucosa may take place as a result of merozoites or sporozoites penetrating the basement membrane of the epithelial border. One frequently observes in sections the parasite occupying the innermost end of the epithelial cell—the part ordinarily occupied by the nucleus; and in the same field one finds other schizonts located in the core of the villus. This seems to suggest that the original infecting elements may not always rest after they have entered the epithelial cells, but in many cases push on to the basement end, and may sometimes even penetrate the basement membrane and enter the subepithelial territory beyond.

The fate of these merozoites, apparently imprisoned in the subepithelial region is uncertain. How they are able to enter the cecal lumen and infect other cells is not clear unless we assume that invasion of the epithelial cells may take place from the region of the submucosa as well as from the lumen of the crypts. In view of the present observations on the location of mature merozoites behind the intact epithelial wall, this view must be held as an open possibility in coccidial infections. This phase of the matter, as well as other details of the pathology of coccidial infections will receive further consideration in the complete paper to appear, with full illustrations at a later date. It might be mentioned, however, that dissemination of merozoites in the individual through the medium of the blood stream is also a possibility. The writer has examined many blood samples with the hope of discovering either merozoites or some of the flagellated organisms, presumably *Trichomonas*, which are found predominantly in the liver lesions in blackhead of turkeys and other birds, as previously reported by the present writer and confirmed by others. They have not yet been observed, however, although bodies simulating the merozoites were discovered in one case in heart's blood and in liver smears. These bodies, eventually proved to be stages in the life history of a blood sporozoon, probably *Haemoproteus*, since they also

occurred in blood cells. The spindle shaped bodies were only about four to five microns in length, considerably smaller than the merozoites of *Eimeria avium*. Blood from the portal circulation has not been examined.

Thus, to conclude, although the full significance of the presence of merozoites and of other stages of *Eimeria avium*, in subepithelial regions of the intestines cannot yet be grasped, their frequency of occurrence there and their freedom from all appearances of degenerative changes lead us to assume that this phenomenon marks an ordinary phase of the normal infective process; and that so far as the coccidia are concerned, we must, as the writer pointed out some years ago, abandon the view that they are exclusive parasites of epithelial cells in the sense that they must occupy epithelial cells to complete their normal development.

REFERENCES

HADLEY 1911 Archiv f. Protistenk., **23**, 7–50.
SMITH, THEOBALD 1916 Jour. Exper. Med., **23**, 293–300.

A BACTERIAL DISEASE OF THE WRAGG CHERRY

PRELIMINARY NOTE

WALTER G. SACKETT

Laboratory of Bacteriology, Colorado Experiment Station, Fort Collins, Colorado

Received for publication, September 30, 1916

During the latter part of June, 1915, the writer received some half-grown, green Wragg cherries, affected with characteristic watery, green spots, which varied in size from tiny specks to irregular splotches involving one-half or more of the cherry. In the more advanced stages, these were slightly sunken, brown to black in color, and watery around the margin.

An examination of the watery tissue showed that bacteria were very abundant, which naturally suggested this group of organisms as a possible cause of the trouble.

Further study has shown that as the disease progresses, the spots increase in size and become sunken, while the tissue involved may shrivel and dry down tight to the pit, so that as the cherry fills out and ripens, one whole side or the end presents a large, sunken, mummified spot, black in the center, with a hard, green border. If many such spots occur, the mature cherry is apt to be very irregular and knotty in outline, and worthless for the market. While this condition is not at all uncommon, we also find many ripe cherries with numerous, hard, sunken, green spots of varying size, a stage, apparently, less advanced than the one just described. As the fruit turns red, new infections appear to be fewer, and the progress of the disease seems to be less rapid; in fact, practically no new lesions can be discovered after the cherries begin to color, and those which appear late on the green cherries are discernible only as small, light discolorations in the ripe fruit.

The stems (peduncles) of the cherries are almost invariably attacked and show the infection by turning black and shriveling. In some cases, they are entirely killed, and the cherries drop

prematurely; again, the movement of food substances and water may be interfered with to such an extent as to dwarf the fruit.

The infection is not confined to the fruit, but involves the leaves and young twigs as well. On the former, there occur numerous, more or less circular, chocolate-brown areas, lighter in the center, from 1 to 7 or 8 mm. in diameter. As the dead tissue in these spots dries, it breaks away from the surrounding green area and ultimately falls out, leaving a hole. As a result of this, badly affected leaves present a typical shothole appearance. Neither premature nor serious defoliation of the trees has been observed to take place.

On the young twigs, we find watery, elliptical, olive-brown discolorations, more or less regular in outline, surrounding the lenticels; these elongate with age and become somewhat sunken and darker in color.

From what has been said thus far concerning the disease, it becomes apparent at once that the symptoms are almost identical with the bacterial disease of the peach and plum, described by Smith, Rorer and Rolfs caused by *Pseudomonas pruni* (*Bacterium pruni*, Smith).

Pure culture isolations on nutrient agar from a varied assortment of diseased cherry tissues have yielded an organism which corresponds essentially to *Pseudomonas pruni*. When reinoculated into suitable cherries, it has produced typical lesions and is, therefore, to be regarded as responsible for the above described disease of the Wragg cherry.

While *Pseudomonas pruni* has been assigned as the cause of a similar trouble on other stone fruits, this is the first time, to the writer's knowledge, that its occurrence has been reported on the cherry, and so far as our present field observations go, the disease has been found only on the Wragg variety, although this statement is only tentative.

Spraying experiments with self-boiled lime-sulphur, conducted during the past spring and summer, reduced the injury to the fruit from 41.4 to 10.2 per cent. Further work along this line is to be carried on during the season of 1917.

A detailed account of the investigation will be published in full later.